Interleukin-2: an accurate biomarker for rapid testing of SARS-CoV-2 vaccine-induced T cell immune responses in whole blood

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Abstract

Background: T cell responses to natural SARS-CoV-2 infection may be more robust and longer lived than antibody responses, thus preventing re-infection. Accurate assessment of vaccine-induced T cell responses is critical for understanding the magnitude and longevity of vaccine-induced immunity across patient cohorts. Aims: To establish a simple, accurate and rapid whole blood test to determine natural and vaccine-induced SARS-CoV-2 immunity via a cytokine release assay. Methods: Cytokine release in whole blood stimulated with peptides specific for SARS-CoV-2 was measured in donors with PCR-confirmed previous infection (n=29), suspected infection (n=30) or with no history of exposure (n=69); and in donors pre- and post-vaccination (n=32). Cytokines were measured by enzyme immunoassay and multiplex array. Results: Cytokines interleukin-2 (IL-2) and interferon-gamma (IFN-γ) were highly elevated in PCR-confirmed or suspected SARS-CoV-2 infected donors at 20->2000pg/ml and 20-1000pg/ml, respectively, compared to history negative controls (<20-90pg/ml). Receiver operating curves showed IL-2 as the superior biomarker with AUC of 0.99 compared to IFN-γ (0.94). Following vaccination, 100% of PCR-confirmed donors and 94% of unexposed individuals demonstrated a positive IL-2 response. Mean IL-2 levels increased ˜18-fold from 12pg/ml pre-vaccination to 202pg/ml and 216pg/ml after the 1 st and 2 nd vaccine doses, respectively. No other cytokines were suitable biomarkers for distinguishing SARS-CoV-2 infection or vaccination responses. Conclusion: This rapid, whole blood-based T cell test can be utilised to make accurate and comparable assessments of vaccine-induced T cell immunity across multiple population cohorts, and aid decision making on public health policies and vaccine efficacy.

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Short title: IL-2 is a biomarker for SARS-CoV-2 T cell immunity

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Conflicts of interest

MAO is an employee of Indoor Biotechnologies, Ltd. BRS and NFB are employees of Indoor Biotechnologies, Inc. MDC is a co-owner and employee of both companies.

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Abstract

Background : T cell responses to natural SARS-CoV-2 infection may be more robust and longer lived than antibody responses, thus preventing re-infection. Accurate assessment of vaccine-induced T cell responses is critical for understanding the magnitude and longevity of vaccine-induced immunity across patient cohorts.

Aims: To establish a simple, accurate and rapid whole blood test to determine natural and vaccine-induced SARS-CoV-2 immunity via a cytokine release assay.

Methods : Cytokine release in whole blood stimulated with peptides specific for SARS-CoV-2

was measured in donors with PCR-confirmed previous infection (n=29), suspected infection (n=30) or with no history of exposure (n=69); and in donors pre- and post-vaccination (n=32). Cytokines were measured by enzyme immunoassay and multiplex array.

Results : Cytokines interleukin-2 (IL-2) and interferon-gamma (IFN-γ) were highly elevated in PCR-confirmed or suspected SARS-CoV-2 infected donors at 20->2000pg/ml and 20-1000pg/ml, respectively, compared to history negative controls (<20-90pg/ml). Receiver operating curves showed IL-2 as the superior biomarker with AUC of 0.99 compared to IFN-γ (0.94). Following vaccination, 100% of PCR-confirmed donors and 94% of unexposed individuals demonstrated a positive IL-2 response. Mean IL-2 levels increased ˜18-fold from 12pg/ml pre-vaccination to 202pg/ml and 216pg/ml after the 1st and 2nd vaccine doses, respectively. No other cytokines were suitable biomarkers for distinguishing SARS-CoV-2 infection or vaccination responses.

Conclusion : This rapid, whole blood-based T cell test can be utilised to make accurate and comparable assessments of vaccine-induced T cell immunity across multiple population cohorts, and aid decision making on public health policies and vaccine efficacy.

Key Words

IL-2, SARS-CoV-2, T cell responses, vaccine-induced immunity, whole blood test,

Abbreviations

AUC – Area under the curve

ELISpot – Enzyme-linked immune absorbent spot

G-CSF – Granulocyte colony-stimulating factor

GM-CSF – Granulocyte-macrophage colony-stimulating factor

IFN-γ – Interferon gamma

IL-10 – Interleukin 10

IL-12p70 – Interleukin 12p70

IL-13 – Interleukin 12

IL-17A - Interleukin 17A

IL-1β – Interleukin 1 beta

IL-2 – Interleukin 2

IL-4 – Interleukin 4

IL-5 – Interleukin 5

IL-6 – Interleukin 6

IL-7 – Interleukin 7

IL-8 – Interleukin 8

MCP-1 – Monocyte chemoattractant protein-1

MERS-CoV – Middle East respiratory syndrome coronavirus

MIP-1β – Macrophage inflammatory protein 1 beta

PCR – Polymerase chain reaction

ROC – receiver-operating characteristic

SARS-CoV-1 – Severe acute respiratory syndrome coronavirus 1

SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2

TNFα – Tumour necrosis factor alpha

Introduction

Understanding the longevity of the adaptive immune response to SARS-CoV-2 is critical for devising public health policies to prevent reinfection. The ability to accurately measure the protective endurance of both memory B and T cells is fundamental to this understanding. Concerns about rapidly waning antibody levels post viral clearance have been raised [1]. However, T cell responses may be more robust and longer lived [2]. Initial studies revealed that virus-specific T cell responses developed in nearly all individuals with confirmed SARS-CoV-2 infection [3-6], with responses persisting for at least six months post-infection[7, 8]. Once vaccination programmes were established, vaccine-induced T cell responses could be investigated. Early studies demonstrated that SARS-CoV-2 vaccines were efficient at generating broad, protective T cell responses [9-12]. Little is known about the longevity of these responses and simple methods for accurately screening viral specific T cell responses at a population level are needed to assess long term vaccine efficacy. Traditional methods of measuring T cell responses are time consuming, difficult to standardise, and require specialised equipment and technical knowledge. Moreover, current commercial tests, such as ELISpot, solely measure T cell production of interferon-gamma (IFN-γ), although other cytokines may provide better indication of anti-viral responses[7]. Simple, 'rapid' tests using whole blood, similar to those routinely used for diagnosis of tuberculosis provide an alternative approach to measure viral specific T cell responses[13-15]. Here, we adapted and optimised an *in vitro* whole blood stimulation assay to determine the most accurate biomarkers for identifying the presence of SARS-CoV-2-specific T cells in naturally infected individuals and in two cohorts of

individuals pre- and post-vaccination. The high specificity and sensitivity of the test distinguishes individuals with either natural and/or vaccine induced T cell immunity from those individuals with no immunity to SARS-CoV-2.

Methods

Study cohort

This study received ethical approval from the Wales Research Ethics Committee 5 (IRAS number: 286991). To investigate T cell responses in naturally infected individuals, participants from across Wales and England were recruited to the project between June-December 2020. All participants gave written, informed consent prior to inclusion. At the time of blood sample collection, corresponding details of prior test results for SARS-CoV-2 infection, confirmed by using PCR with reverse transcription from a nose and throat swab performed by public health bodies at accredited laboratories were obtained via questionnaire. Those that had a positive PCR test were assigned to the 'Confirmed infection (PCR positive') cohort (n=29). Participants were also asked if they had ever tested positive for SARS-CoV-2 via a lateral flow test, and to note any COVID-19-related symptoms they had experienced as well as record any close contact they had with persons who had been confirmed positive by PCR. Those that had a positive lateral flow test, experienced COVID-19-like symptoms or had close contact with a positive individual were assigned to the 'Suspected infection/exposure' cohort (n=30). All other individuals were assigned to the 'No history of exposure' cohort (n=69). None of the participants were hospitalised due to COVID-19 at any time before or during the study.

To investigate T cell responses in vaccinated individuals, participants from across Wales, England and the US were recruited to the project between December 2020 and March 2021. All participants gave written, informed consent prior to inclusion. At the time of blood sample collection, details of prior test results for SARS-CoV-2 infection and details of SARS-CoV-2 vaccinations including date and vaccine manufacture were obtained via questionnaire: 26 donors received the Pfizer-BioNTech vaccine, 5 received the Oxford-AstraZeneca vaccine and 1 received the Johnson and Johnson/Janssen vaccine.

Peptides

The SARS-CoV-2 peptide pool consisted of 470 15mer peptides overlapping by 11-amino acids, covering the entire proteome of the nucleocapsid phosphoprotein (Miltenyi Biotec, Bergisch Gladbach, Germany), membrane glycoprotein (Miltenyi Biotec) and the spike (S1 and S2) protein (JPT Peptide Technologies, Berlin, Germany). All peptides were purified to >70% by HPLC and used at a final concentration of 0.5µg/ml/peptide.

Stimulation

A single 10ml sodium heparin vacutainer (BD) tube of blood was collected from each participant and processed in the laboratory within 12 hours of blood draw. Whole blood samples (1ml) were aliquoted into sterile T332 Micrewtubes (Simport Scientific, Saint-Mathieu-de-Beloeil, Canada) containing pre-aliquoted peptides. One tube contained the T cell mitogen, phytohaemagglutinin-L (Sigma-Aldrich, St. Louis, Missouri, USA; final concentration 50µg/ml) as a positive control, and another contained 50µl PBS (negative control). Samples were incubated at 37°C, 5% CO_2, for 18-22 hours. Tubes were then centrifuged at 2000g for 3 minutes before harvesting ˜200-300µl plasma from the top of each blood sample. Plasma samples were stored at -20°C for up to 6 weeks prior to analysis by ELISA or Luminex xMAP array.

ELISA for IFN-γ

IFN-γ protein was measured by a commercially available IFN-γ ELISA MAX Deluxe kit (BioLegend, San Diego, California, USA), following manufacturer's instructions with a few modifications: an additional point on the standard curve (1000pg/ml); a 1-hour incubation for standards, samples and blanks; and a pre-read step (at 450nm with just the TMB substrate) to standardise development time of the assay. When standard 2 reached 0.1 OD, stop solution was added and the plate was read at 450nm. The amount of IFN-γ in each sample was analysed using the Gen5 software 8-point standard curve. To calculate the T cell response to

SARS-CoV-2, the amount of IFN-γ in the control (PBS only) sample was subtracted from the corresponding value for the SARS-CoV-2 peptide stimulated sample and reported as pg/ml of plasma. In the absence of a response to the peptides, the amount of IFN-γ was calculated as below the lower limit of detection (LLOD). Therefore, a value of the lowest value on the standard curve was given for that sample (7.813pg/ml). Cut off for determining a positive response was set by Youden's index (J) (see *Statistics* below) which determined the optimal cut off value of >24.04pg/ml in the test for natural infection-induced responses, and >42.34pg/ml in the test for vaccine-induced responses.

Luminex cytokine array

Protein levels of IL-2, TNFα, MIP-1β, MCP-1, IL-1β, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13 IL-17A, G-CSF and GM-CSF were measured by commercially available Human Cytokine 17-plex Assays and Human Cytokine Th1/Th2 Assays (Bio-Rad, Hercules, CA, USA), following manufacturer's instructions. The median fluorescent intensity (MFI) of each cytokine bead set was measured on a Bio-plex 200 instrument (Bio-Rad). Cytokine concentration was calculated from control curves of standards provided in the kit. In the absence of a response to the peptides, the amount of cytokine was considered as below the LLOD. For IL-2, a value of the lowest value on the standard curve was given for that sample (6.28pg/ml). For all other cytokines, this was recorded as out of range. If a response was greater than the highest point on the standard curve, this was also recorded as out of range. As above, cut off for determining a positive response was set by Youden's index. The IL-2 optimal cut off was determined as >19.91pg/ml in the test for natural infection-induced responses. For the test for vaccine-induced responses, optimal cut off values for IL-2, IL-13 and IL-10 were >37.08pg /ml, >2.28pg/ml and >7.81pg/ml, respectively.

Statistics

GraphPad Prism Version 9.0.1 was used for all statistical analyses of datasets.

Significance was determined using one-way ANOVA tests with multiple comparisons being made using Tukey's multiple comparison test; a P-value <0.05 was considered significant. ROC curves and corresponding sensitivity and specificity values were generating from the ROC analysis on GraphPad Prism. As a summary measure of the ROC curves, the Youden Index (J) was used to enable the selection of a threshold value (cut off point for positivity) for that marker, whist also determining both sensitivity and specificity of each biomarker [16, 17]. Pearson R squared values and associated p values were calculated by correlation analysis on GraphPad Prism.

Results

Cytokine profiles of SARS-Cov-2 infected and uninfected individuals

Blood samples from 128 participants were stimulated overnight with a mega pool of 470 SARS-CoV-2 peptides and the level of IFN-γ was measured in the plasma (see Methods, Online Repository). Significant differences were observed in the magnitude of IFN-γ responses between individuals with previous PCR-confirmed infection and suspected infection (mean IFN-γ = 274pg/ml and 145pg/ml, respectively) and individuals with no history of exposure (mean IFN-γ = 26pg/ml) (Figure 1A). In the cohorts of PCR positive individuals and those with suspected infection/exposure, 97% of individuals (28/29) and 80% of individuals (24/30) respectively demonstrated a positive response. This was in comparison to 20% (14/69) of donors from the cohort of unexposed individuals. A positive response (>24pg/ml) was determined by Youden's Index (see Methods).

Additional SARS-CoV-2-induced cytokines were measured by multiplex array (see Methods) to determine whether other biomarkers could more accurately distinguish natural infection from non-infection. A clear distinction in the magnitude of interleukin-2 (IL-2) responses was observed between individuals with previous PCR-confirmed infection and suspected infection (mean IL-2 = 328pg/ml and 275pg/ml, respectively) and individuals with no known history of exposure (mean IL-2 = 9pg/ml) (Figure 1B). From the cohort of previously infected donors, all 29 individuals demonstrated a positive IL-2 response, in marked contrast to

only 1/69 from the unexposed cohort. In the cohort with suspected infection/exposure, 77% of individuals demonstrated a positive response (>19.9pg/ml).

Receiver-operating characteristic (ROC) curves were generated for IL-2 and IFN-γ. For IL-2, the area under the curve (AUC) value was 0.9950 (P <0.0001) (Figure 2A). For IFN-γ, the AUC value was 0.9465 with a P value of <0.0001 (Figure 2B). Additionally, Youden's Index was used to measure the potential effectiveness of each biomarker. For IL-2, a sensitivity of 98.6% and a specificity of 100% was achieved. For IFN-γ, a sensitivity of 79.7% and specificity of 96.6% was attained.

Significant differences were also observed for interleukin-13 (IL-13) and interleukin-6 (IL-6) between the PCR positive and uninfected groups (Supporting Figure 1 A-B). Neither were as accurate as IL-2 or IFN-γ at differentiating between cohorts (AUC = 0.6669 for IL-13 and 0.6717 for IL-6, Supporting Figure 1 C-D). No other cytokine examined could discriminate between previously infected and unexposed individuals (TNFα, MIP-1β, IL-12p70, IL-4, IL-5, IL-10, G-CSF, IL-17A, IL-7, IL-8, IL-1β, MCP-1, GM-CSF - data not shown).

These results demonstrate unequivocally that IL-2 is the most accurate biomarker for distinguishing SARS-CoV-2 infected from uninfected individuals.

Cytokine response following vaccination

To investigate whether IL-2 was also an accurate biomarker for identifying vaccine-induced T cell responses, 32 individuals were recruited to donate blood samples prior to SARS-CoV-2 vaccination and following the first and/or second vaccine doses (see Methods for vaccine details).

A marked and consistent increase in the magnitude of IL-2 responses was observed between pre- and post-vaccination in previously unexposed individuals. The mean IL-2 response increased by ˜18-fold from 12pg/ml prior to vaccination to 203pg/ml and 216pg/ml after the 1st and 2ndvaccine doses, respectively (Figure 3A). Following vaccination, 94% of individuals demonstrated a positive IL-2 response (>37.1pg/ml).

In the cohort of previously infected individuals, all donors demonstrated a positive IL-2 response following the 1st and 2nd doses of vaccine (Figure 3B). Vaccination did not boost the magnitude of responses from the levels of IL-2 seen pre-vaccination, and the mean level of IL-2 produced by these donors, although slightly higher than the cohort with no previous exposure, was not significantly increased (277pg/ml vs 209pg/ml).

An ROC curve was generated from the data obtained from previously unexposed individuals (Figure 3C). The AUC value was 0.9896 (P <0.0001). The test sensitivity was 94.3% and the specificity was 95.5%.

Significant differences were similarly observed in the magnitude of IFN-γ responses between pre- and post-vaccination in previously unexposed individuals (Figure 4A). Mean IFN-γ levels increased by ˜10-fold following vaccination. In the pre-vaccinated group, 5% of individuals demonstrated a positive IFN-γ response (>42.3pg/ml), in noticeable contrast to 88% and 81% following 1 or 2 vaccine doses. A sensitivity of 84.9% and specificity of 95.2% was achieved (Figure 4B).

The magnitude of the IL-13 response was small (<10pg/ml), but the differences between pre- and post-vaccination were significant (Figure 5A). After the 1st vaccine dose, 85% of donors showed a positive IL-13 response (>2.3pg/ml), dropping to 67% in donors having received 2 doses. The magnitude of the IL-10 response was again small, but a significant difference was observed between pre-vaccinated samples, and samples taken after the 2nd vaccine dose only (Figure 5B). All samples gave a positive IL-10 result following 2 vaccinations, however, so did 31% of the unvaccinated samples (>7.8pg/ml). Cytokines TNFα, IL-12p70, IL-4, IL-5, and GM-CSF were also measured but none were effective at differentiating unvaccinated from vaccinated individuals (data not shown).

These results confirm that IL-2 is the most accurate biomarker for distinguishing unvaccinated and vaccine-induced T cell responses.

Finally, we evaluated whether there was any correlation between the magnitude of the vaccine-induced IL-2 response and the number of days that had passed since receiving the second vaccine. The IL-2 response

remained elevated (>20pg/ml) up to 80 days post-vaccination and did not significantly decrease over time ($p = 0.224$; Figure 6). This suggests that within ˜80 days, the vaccine-induced T cell response is not waning.

Discussion

Simple, rapid and functional assays that accurately detect SARS-CoV-2-specific T cell responses are essential for comparing T cell immunity across multiple population cohorts and for long-term assessments of vaccine efficacy. The results show that measuring plasma IL-2 from SARS-CoV-2 peptide-stimulated whole blood accurately distinguishes between COVID-19 convalescents and uninfected healthy blood donors with a high degree of sensitivity and specificity. Plasma IL-2 was also an excellent biomarker for distinguishing vaccinated and unvaccinated individuals. Furthermore, T cell responses in previously unexposed individuals developed early after just one vaccination, were maintained after a second dose and were comparable to those with a history of SARS-CoV-2 infection; a finding that was not observed using ELISpot [18, 19]. Positive IL-2 responses were still detectable up to 78 days post-vaccination, in keeping with observations that T cell responses are robust; in SARS-CoV-1 and MERS-CoV infected individuals, T cell responses were still identifiable several years after infection [20-22].

Reliable biomarkers are integral to assessing vaccine efficiency over time. Unlike other immunoassays that detect antigen-specific T cell responses such as ELISPOT and flow cytometry, this virus-specific IL-2 release assay is simple to perform and can be employed across multiple laboratories for large scale epidemiological studies in conjunction with testing for neutralising antibodies [23]. The IL-2 release assay is a promising tool for use in multi-centre clinical trials for the development of new vaccines or treatments against novel SARS-CoV-2 variants, such as the delta variant, as they arise globally. Further studies of this approach should aid decision making by health policy makers, including vaccine booster requirements and monitoring of SARS-CoV-2 infection in immune-supressed and other at-risk populations.

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Figure Legends

Figure 1. Measurement of cytokine productionto determine SARS-CoV-2-specific T cell responses in naturally infected and non-exposed individuals.

(A) IFN-γ and (B) IL-2 release in response to overnight stimulation with SARS-CoV-2 peptide mega pool in PCR-positive (*), suspected infection (*), and no known history of exposure (*) cohorts. Results show arithmetic mean +/-SEM. Dashed line = positivity cut off. *p<0.05, **p<0.01, ****p<0.0001, ns = not significant.

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Receiver operating characteristic curves defining sensitivity and specificity readouts for IL-2 (n=49) (A) and IFN-γ (n = 59) (B) are shown. Area under the curve (AUC) and associated P value are indicated.

Figure 3. Measurement of IL-2 production to determine SARS-CoV-2-specific T cell responses in individuals pre- and post-vaccination.

IL-2 release in response to overnight stimulation with the SARS-CoV-2 peptide pool in unexposed (A) and previously infected (B) individuals pre- and post-vaccination (*=pre-vaccination, *=1stvaccination, *=2nd vaccination). Square symbol=Janssen vaccine (A only). Results show arithmetic mean +/-SEM. Dashed line = positivity cut off. (C) ROC curves defining sensitivity and specificity for IL-2. AUC and associated P value are indicated.

Figure 4. Measurement of IFN-γ production to determine SARS-CoV-2-specific T cell responses in individuals pre- and post-vaccination.

(A) IFN-γ release in response to overnight stimulation with the SARS-CoV-2 peptide pool in previously unexposed individuals pre- and post-vaccination (*=pre-vaccination, *=1stvaccination, *=2nd vaccination). Square symbol=Janssen vaccine (A only). Results show arithmetic mean +/-SEM. Dashed line = positivity cut off. *p<0.05, **p<0.01, ns = not significant. (B) ROC curves defining sensitivity and specificity for IFN-γ. AUC and associated P value are indicated.

Figure 5. Measurement of IL-13 and IL-10 production to determine SARS-CoV-2-specific T cell responses in individuals pre- and post-vaccination.

IL-13 (A) and IL-10 (B) release in response to overnight stimulation with the SARS-CoV-2 peptide pool in previously unexposed individuals pre- and post-vaccination (*=pre-vaccination, *=1stvaccination, *=2nd vaccination). Results show arithmetic mean +/-SEM. Dashed line = positivity cut off. *p<0.05, **p<0.01, ns = not significant.

Figure 6. Correlation analysis of IL-2 response with number of days since last vaccine administration

The relationship between IL-2 production and the number of days post vaccination was evaluated. R squared and p values are indicated. Each symbol represents an individual donor.

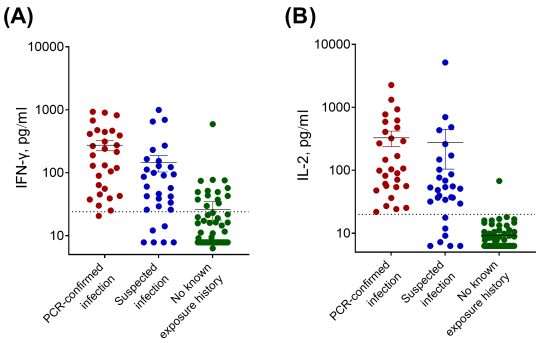

Figure 1_Oliver et al.

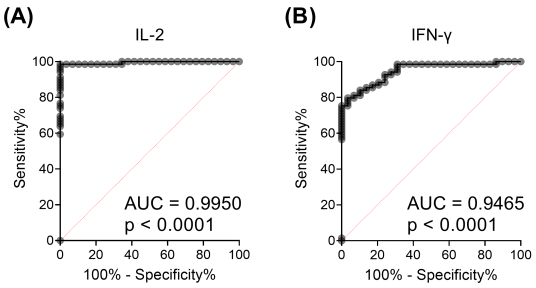

Figure 2_Oliver et al.

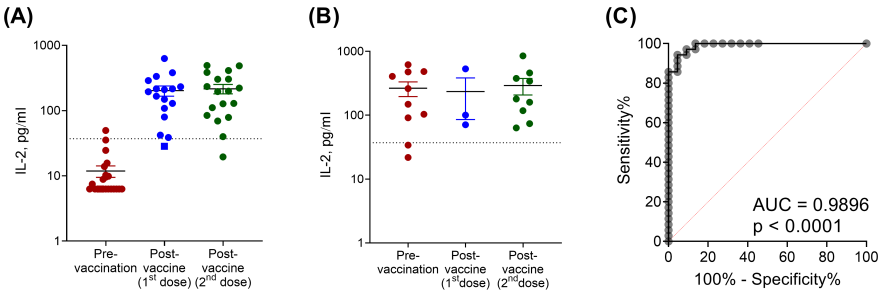

Figure 3_Oliver et al.

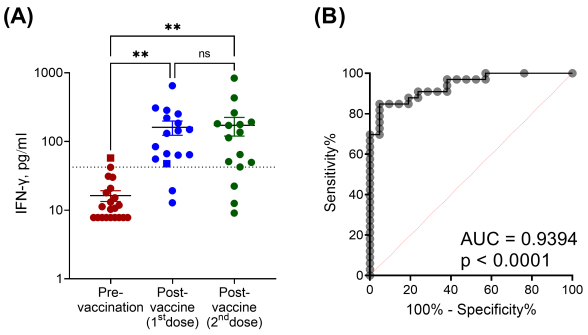

Figure 4_Oliver et al.

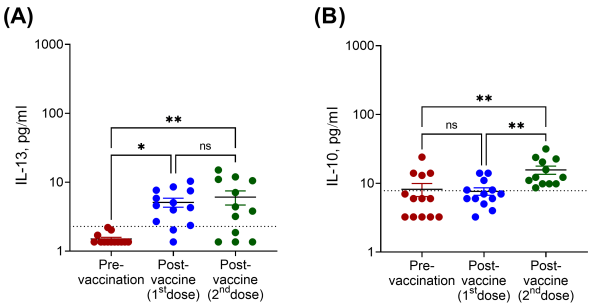

Figure 5_Oliver et al.

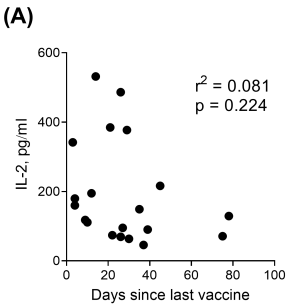

Figure 6_Oliver et al.