Early transcriptional responses following Ad26.COV2.S vaccination in individuals with prior SARS-CoV-2 infection

Veronica V. Rezelj,[a,c] Jeroen Tolboom,[b,c] Nina Hertoghs,[a] Jan Serroyen,[b] Mathieu Le Gars,[a] Frank Struyf,[b] Jenny Hendriks,[a] Javier Ruiz Guiñazú,[b] and Chelsea McLean[a,*]

[a]Janssen Vaccines and Prevention, Leiden, the Netherlands
[b]Janssen Research and Development, Beerse, Belgium

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Summary

Background Ad26.COV2.S is a recombinant, replication-incompetent human adenovirus serotype 26 (Ad26) vectored vaccine encoding a full-length SARS-CoV-2 spike protein in a prefusion-stabilised conformation. The genes and pathways which are differentially regulated following vaccination with Ad26.COV2.S, how these differ over time, and under different regimens, have not been investigated.

Methods Gene expression changes were profiled via RNAseq following the first and second dose of Ad26.COV2.S in a subset of individuals with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection, included in a randomised, double-blind, Phase 3 study (NCT#04908722). Transcriptional responses induced by a 1- and 2-dose regimen were assessed, each at two different dose levels: the marketed 5×10^{10}vp dose level and a lower 1.25×10^{10}vp dose level.

Findings Consistent with other vaccine studies, antiviral, interferon, cytokine, and monocyte blood transcriptional modules were differentially regulated one day post-Ad26.COV2.S vaccination. At three days post-vaccination, innate immunity was still transcriptionally active at lower levels than one day post-vaccination. By seven days post-Ad26.COV2.S, increased immunoglobulin gene expression dominated the transcriptome response, indicating a shift towards humoural and B cell responses and activation of adaptive immunity. Transcriptional responses post-Ad26.COV2.S were generally stronger after the first dose versus the second dose.

Interpretation In this study in whole blood, innate and adaptive immune transcripts were upregulated as early as one and seven days post-Ad26.COV2.S, respectively. These findings add to the limited data on innate immune responses to adenovirus-based vaccines in a clinical setting and contribute to understanding the mechanisms behind the adaptive immune responses that may contribute to protective immunity.

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Keywords: Vaccination; RNAseq; Whole blood; Transcriptome; COVID-19; Ad26.COV.2.S

Introduction

Vaccine-induced markers of humoural and cellular immunogenicity are generally well studied. The advent of 'omics' technologies has provided valuable insights into the molecular mechanisms that orchestrate such vaccine-induced responses. Among these technologies, transcriptomics has emerged as a valuable tool in the field of vaccines.

Specifically, transcriptomics has proven instrumental in identifying potential predictors of immunity, unravelling pathways, and elucidating biological networks associated with vaccine-induced adaptive immunity. The correlation between early gene expression signatures and humoural responses after immunisation are particularly noteworthy, and were demonstrated for various vaccines, including investigational universal[1] or licenced[2,3] influenza vaccines, Ebola,[4] and a yellow fever[5] vaccine. Moreover, the comparison of transcriptional signatures across diverse vaccines has revealed both commonalities and distinct patterns after immunisation.[6,7] Thus, characterisation of vaccine-induced transcriptional responses can (i) offer unique

*Corresponding author.
E-mail address: CMcLean@its.jnj.com (C. McLean).
[c]Co-first authors.

Research in context

Evidence before this study

We conducted a PubMed search for research articles published from database inception to 12 November, 2025 using key search terms "adenovirus 26" AND "vaccine" with no language restrictions. Search results were then filtered to display results from "randomised controlled trials" only. A total of 60 articles were retrieved. Of the articles retrieved, seven articles investigated the Ad26-vectored respiratory syncytial virus (RSV) vaccine, three investigated the Ad26-vectored COVID-19 vaccine, and seven investigated the Ad26-vectored Ebola vaccine. When the search term "transcriptomics" was added, no results were retrieved, highlighting the gap in current literature on the transcriptomic response following Ad26-vectored vaccines. A second exploratory search was then conducted, using the search terms "vaccine" AND "transcriptome" and filtering for results from "clinical trials" only, and 131 articles were retrieved. Among these were four articles which reported transcriptomic data on vaccines within similar disease settings. One article described the immune response following infection with the SARS-CoV-2 virus, using samples from two previously conducted randomised placebo-controlled trials. This article reported a decrease in inflammatory signatures following vaccination, which supports a vaccine-mediated benefit with respect to breakthrough infections. The second article reported on a phase 1 study in Chinese adults administered an inactivated SARS-CoV-2 vaccine and identified a large number of genes related to the vaccine-induced immune response. The third article compared the transcriptional response in elderly participants administered a fractional dose of a COVID-19 vaccine either intradermally or intramuscularly and found no difference in the transcriptomic response induced by the two administration routes. The fourth article described a multi-omics signature, consistent with persistent inflammation driving anaemia and stress erythropoiesis, which was associated with increased risk of long COVID.

Notably, when all filters were removed, several relevant articles were identified, including one article which reported on the transcriptomic response following the Ad26-vectored Ebola vaccine, but no articles reported on the transcriptional response following the Ad26 vectored COVID-19 vaccine. In the context of vaccines targeting COVID-19, the existing evidence in the literature indicates that the transcriptional response varies based on vaccine regimen and number of doses administered. For example, one study performed a comprehensive transcriptional analysis and comparison of 13 different vaccines and their antibody responses and reported that the whole blood transcriptome following primary vaccination with mRNA COVID-19 vaccines and the adenovirus-vectored ChAdOx1-S COVID-19 vaccine is similar. A further study comparing two MVA-vectored SARS-CoV-2 vaccines, encoding either the native or the pre-fusion stabilised spike protein, indicated that the transcriptional response to both vaccines was similar in composition, but was higher in magnitude with the prefusion-stabilised spike protein. Two additional studies reported that a second vaccination with mRNA COVID-19 vaccines elicits a greater transcriptional response compared to the first vaccination, and is characterised by early activation of innate, antiviral, and interferon pathways. Furthermore, a study reporting on a self-amplifying mRNA COVID-19 vaccine identified links between the early (innate) post-vaccination transcriptional response and later post-vaccination (adaptive) antibody titres.

Added value of this study

Currently, the genes and pathways which are up- or down-regulated following vaccination with Ad26.COV2.S, how these differ over time and under different regimens, and whether these correlate with antibody response have not been investigated.

In the context of a Phase 3 study (NCT#04908722), both a 1-dose and 2-dose Ad26.COV2.S regimen were administered, each at two dose levels: the marketed 5×10^{10}vp dose level and at a lower dose level of 1.25×10^{10}vp. Samples collected from a subset of 74 participants within this study, with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection, enabled analysis of the early (up to one week post-vaccination) transcriptional response, as well as the antibody response at 28-days post-vaccination.

The results reported here provide a comprehensive profiling of Ad26.COV2.S-induced early transcriptional responses, and their associated antibody responses.

Implications of all the available evidence

Ad26.COV2.S upregulated innate immune response genes as early as 1 Day Post-Vaccination while adaptive immune response genes exhibited up-regulation from 7 Days Post-Vaccination. These findings align with previous studies investigating transcriptional responses following COVID-19 vaccination(s) against other unrelated, supporting that initiation of innate and adaptive immune transcriptional responses is generalisable across a range of pathogens. Furthermore, our results show that while transcriptional responses were similar across different dose levels of the vaccine, transcriptional responses following Ad26.COV2.S vaccination were generally stronger after the first dose compared to the second dose. These results were consistent with the observed humoural immune responses, since a second dose of Ad26.COV2.S did not elicit a further increase in S-binding antibody levels in the studied population who had pre-existing immunity to SARS-CoV-2 due to prior SARS-CoV-2 infection.

This is unlikely to be due to the induction of antibodies directed against the Ad26 vector, as a second dose of Ad26.COV2.S at 5×10^{10}vp, administered with the same interval as in this study, was previously demonstrated to elicit a stronger binding antibody response than the first dose in adults without pre-existing immunity.

The difference between our results and those of other studies with different COVID-19 vaccines can likely be attributed to the SARS-CoV-2 exposure history of the populations examined in those studies. While our analysis focused on a population with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection, the published studies were conducted on individuals without previous exposure to the virus.
Therefore, future COVID-19 vaccine studies should take into account the exposure history of the target population during study design to ensure the desired demographics in terms of previous COVID-19 exposure and to facilitate interpretation of results.

insights into the underlying mechanisms that contribute to a robust and potentially protective immune response and (ii) support the refinement of future vaccine development and design.

In the context of vaccines targeting coronavirus disease 2019 (COVID-19), studies have demonstrated that the whole blood transcriptome following vaccination with mRNA vaccines or the adenovirus-vector ChAdOx1-S vaccine was very similar.[8] It was also observed that a second vaccination with mRNA vaccines elicits a greater transcriptional response compared to the first vaccination, characterised by early activation of innate, antiviral, and interferon pathways.[9,10] Additionally, studies have revealed that transcripts involved in toll-like receptor signalling, antigen presentation, and complement activation one day post-vaccination are associated with higher antibody titres after vaccination with an investigational self-amplifying COVID-19 vaccine.[11]

Ad26.COV2.S, is a recombinant, replication-incompetent human adenovirus serotype 26 (Ad26) vector encoding a full-length SARS-CoV-2 spike (S) protein in a prefusion-stabilised conformation.[12,13] Currently, the genes and pathways which are up- or down-regulated following vaccination with Ad26.COV2.S, and how these differ over time and under different regimens, have not been investigated. Our goal in this study was to comprehensively profile Ad26.COV2.S-induced early transcriptional responses. Using RNA sequencing, we examined and compared gene expression following the first and second dose of Ad26.COV2.S in a subset of individuals with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection, included in a randomised, double-blind, Phase 3 (NCT#04908722) study aimed at evaluating the end-of-shelf life of the vaccine.[14] We compared transcriptional responses induced by a 1- and 2-dose regimen, each at two different dose levels: the marketed 5×10^{10}vp dose level and at a lower dose level of 1.25×10^{10}vp.

Methods

As part of the Sub-study in the VAC31518COV3003 clinical trial, whole blood (2.5 mL whole blood in PAXgene RNA tubes) was collected pre-vaccination and at different times post-vaccination from COVID-19 vaccine naïve participants who were either SARS-CoV-2 seronegative or SARS-CoV-2 seropositive at baseline, as determined by local fingerpick serology testing.

The collection of these tubes was intended to examine gene expression (transcriptional responses) at different time points after one and two vaccinations with Ad26.COV2.S at different dose levels, by RNA sequencing.

Ethics

The VAC31518COV3003 parent study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards (or Ethics Committees) as listed in the Supplementary Materials.

Informed consent was obtained from all subjects involved in the study. All participants included in this report provided written informed consent for the testing described herein.

Power calculation

No formal power calculation was performed, given that thousands of genes were quantified and tested for differential expression simultaneously and the lack of available historical data. The original sample size of 40 SARS-CoV-2 seronegative and 20 SARS-CoV-2 seropositive participants per group was selected with the intention of allowing for a descriptive analysis of gene expression profiles in the subgroup of interest and was limited by the minimum number of available participants per subgroup by design, i.e., the number of SARS-CoV-2 seropositive at baseline (N = 20) when the sample selection was conducted.

Randomisation and handling of missing data

Randomisation occurred in the parent COV3003 Phase 3 study, and RNA extraction and RNAseq were conducted blinded to group. Missing data were not imputed.

Participant and sample selection

In the VAC31518COV3003 Sub-study, four different groups of participants were recruited and were administered two vaccinations of one of four dose levels of Ad26.COV2.S, as detailed in Supplementary Fig. S1A. Participants were enrolled as SARS-CoV-2

seronegative (40 participants planned) or SARS-CoV-2 seropositive (20 participants planned) at baseline, which was determined by local fingerprick serology testing at baseline at the time of enrolment.

Whole blood samples in PAXgene tubes (Qiagen, catalogue #762165) were collected at pre- and post-vaccination time points. As shown in Supplementary Fig. S1B, pre-vaccination sampling occurred on Day 1 (Pre-Vaccination 1) and Day 57 (Pre-Vaccination 2). Post-vaccination sampling took place on Days 2, 4 and 8 (1, 3, and 7 days Post-Vaccination 1) and on Days 58, 60 and 64 (1, 3, and 7 days Post-Vaccination 2).

The selection of SARS-CoV-2 seronegative or seropositive at baseline participants was based on serostatus assignment using results from local fingerpick serology testing that were used for screening, and which were available at the time of participant selection. However, the pre-specified gene expression analysis plan stated that the general serostatus at baseline definition applied in the COV3003 study, which is based on central testing, namely Spike and/or Nucleocapsid-based serology, was used here as well.

Upon unblinding of Study COV3003 (at the time of Primary Analysis), central testing results became available. It was found that out of a total of 73 participants in the RNAseq subset whose samples passed QC and were included in the RNAseq analysis, 1 (1.4% %) participant was SARS-CoV-2 seronegative at baseline based on central testing. Therefore, 38 out of 38 (100%) and 34 out of 35 (97%) participants in groups receiving 2 doses of Ad26.COV2.S at the 5 × 1010vp or 1.25 × 1010vp dose level, respectively, were SARS-CoV-2 seropositive at baseline. This finding rendered the objective to compare the transcriptional response over time in participants who are SARS-CoV-2 seronegative at baseline and those that are SARS-CoV-2 seropositive at baseline non-achievable. Therefore, the planned analyses and objectives were performed only on participants who were SARS-CoV-2 seropositive at baseline.

If participants were identified as having experienced a SARS-CoV-2 infection between Day 1 and Day 64, data derived from these participants from the time of having experienced the infection was excluded from the analysis. SARS-CoV-2 infections were identified as in the COV3003 clinical trial: i.e., by reported PCR or N serology seroconversion, with none identified in the RNAseq subset.

RNA extraction and library preparation

RNA extraction and Next Generation Sequencing was performed in a blinded fashion by Eurofins. Total RNA was extracted from 4 mL whole blood in PAXgene RNA tubes using the Qiagen PAXgene Blood RNA extraction kit (catalogue #762174) and the QIAcube instrument.

RNA quantification was performed by Invitrogen Qubit and a QC assessment carried out with Agilent Bioanalyser. Isolated RNA that passed QC included those for which the RNA content was >200 ng and with sufficient quality (RNA integrity scores ≥6). Samples passing QC were brought forward for library preparation. Seventeen samples did not pass the QC. A limited number of samples (N = 8/17) were brought forward for sequencing despite not meeting the lower input limit (200 ng) recommended for Eurofins, if the RNA integrity score was ≥6, and nine out of the 17 samples failing QC were not sequenced. This resulted in a total of 72 samples at Day 1, Day 2, and Day 64; 67 samples at Day 4; 69 samples at Day 8 and Day 57; and 65 samples at Day 58 and Day 60.

Library preparation was performed using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina® with polyA enrichment (catalogue #E7760L) according to Eurofins SOP. The Qiagen QIAseq FastSelect Globin kit (catalogue #10402478) was used for globin depletion following the manufacturer's instructions.

Libraries were quantified by Invitrogen Qubit, QC checked with the Agilent Bioanalyser, normalised, and paired-end sequenced on an Illumina Novaseq, with 150 bp reads and a target of 40 M read pairs (80 million total reads) per sample.

Statistical analysis

RNA-seq read mapping

Read mapping was performed within the Johnson & Johnson Data Science NF-Tower environment (version 22.10.7) using the RNAseq Pipeline (https://github.com/nf-core/rnaseq), which includes the following steps. Merged FASTQ reads were trimmed to remove adaptors and mapped to the reference genome using STAR. The reference genome used was the Ensembl (GRCh38 primary assembly of the human genome, https://ftp.ensembl.org/pub/release-108/fasta/homo_sapiens/dna/Homo_sapiens.GRCh38.dna.primary_assembly.fa.gz). Quantification was done using RSEM (RNA-Seq by Expectation Maximisation), which utilises an expectation-maximisation algorithm to effectively use ambiguously-mapping reads for gene-level abundance estimates. From the RSEM output, the expected counts per gene were used for downstream analysis. QC metrics were calculated for the reads and the mappings using MultiQC and included an evaluation of the number of total reads, percentage of mapped reads, genomic origin of the reads (exonic versus intronic versus intergenic), gene coverage profiles, and 5'-3' bias.

Differential gene expression analysis

To explore potential confounding effects, different models (including all participants) were run, each containing a single secondary covariate of interest (e.g., Spike antibody levels, age, sex, or BMI) in addition to the primary covariate of treatment (vaccine dose level). As confounding effects were largely absent,

downstream analyses did not include additional covariates in the model.

Differential gene expression analysis was performed using the transcriptomicsTemplates R package within the RNAseq Pipeline of the Johnson & Johnson Data Science NF-Tower environment (version 22.10.7). The filterByExpr function from EdgeR was used to filter out lowly expressed genes. Limma, with voom transformation, was used to quantify the expression level of each transcript. The library sizes were normalised by using the TMM (Trimmed Mean of M values) normalisation method available in EdgeR. Limma was used to produce fold changes from baseline for contrasts of interest.

A placebo control group was not available in this study, so comparisons were made between each time point Post-Dose 1 vaccination and baseline (D1), and between each time point Post-Dose 2 vaccination and Pre-Dose 2 (D57). For cases where time points were not directly compared to baseline (e.g., Post-Vaccination 1 versus Post-Vaccination 2 comparison), the comparisons are also adjusted for subject effect in the statistical analysis.

Given the insufficient number of seronegative at baseline participants, the comparisons were performed with a focus on seropositive at baseline participants and the single participant who was seronegative at baseline was excluded from the analysis.

A gene was considered to be differentially expressed if the false discovery rate (FDR) corrected p-value was <0.05 and the absolute fold change was >1.5 (i.e., absolute log2 fold change >0.58).

BloodGen3 blood transcription module (BTM) analysis

A BloodGen3 BTM analysis was performed on each gene list that came out of the differential gene expression analysis. This analysis allows visualisation of results from the DEG analysis at the module level, representing the percentage of up-regulated and down-regulated module genes for a given module.

The BTM analysis was performed considering only genes which were greater than 1.5 fold (≥0.58 Log_2 fold change) differentially regulated (upregulated or down-regulated) and with an FDR corrected p-value less than 0.05.

Differentially expressed genes were ranked based on adjusted p-value. For the BloodGen3 BTM analysis described above, a gene was considered differentially expressed if the false discovery rate (FDR)-adjusted p-value is less than 0.05 and the fold change >1.5 (|Log2 FC| > 0.58, FDR-adjusted p value < 0.05). BTMs identified as significant in the BloodGen3 BTM analysis are considered as being differentially regulated based on at least 5% of the genes in a module being differentially expressed in the same direction. For this study, only BTMs having at least 50% of the genes in a module differentially expressed in the same direction are reported.

BTM GSVA (gene set variation analysis) analysis

An additional gene set/pathway analysis, specifically a gene set variation analysis (GSVA) was used to corroborate the BloodGen3 analysis results with a different method.

GSVA is a type of gene set enrichment analysis that functions on the single sample level and uses gene sets, rather than individual genes, as the functional unit. Therefore, the output of a GSVA analysis is an estimate of gene set activity.

This is achieved by transforming the traditional input of a gene-by-sample expression data matrix into a corresponding gene-set-by-sample expression data matrix. The resulting gene-set-by-sample expression matrix can be then used with various classical analytical methods, including differential expression, in a gene-set (rather than individual gene) centric manner.

The GSVA method also allows for sample-level comparisons between gene expression and pathway data.

A gene set was considered to be differentially expressed if the false discovery rate (FDR) corrected p-value was <0.05.

Role of funders

This study was exclusively funded by Johnson & Johnson Innovative Medicine. Employees of Johnson & Johnson Innovative Medicine were responsible for the study design, data collection, data analyses, interpretation, and writing of this report.

Results

RNAseq subset baseline characteristics

Participants in the RNAseq subset (N = 74) of the clinical study COV3003[14] included adults 18–55 years of age who received 2 doses of Ad26.COV2.S, administered two months apart, at either a 5×10^{10}vp (the marketed vaccine dose level) or a 1.25×10^{10}vp dose level (Supplementary Fig. S1). Baseline and demographic characteristics of participants in the RNAseq subset were well balanced across the 5×10^{10}vp and 1.25×10^{10}vp groups (Supplementary Table S1). All participants in the subset were SARS-CoV-2 seropositive at baseline, as determined by a positive result by either validated S-ELISA and/or Nucleocapsid (N) serology by Roche elecsys at baseline (Day 1) (Fig. 1). Participants with infections during the course of the study were defined based on N-serology seroconversion. SARS-CoV-2 nucleocapsid protein (N)-specific binding antibodies were measured using the validated Roche elecsys assay (by Labcorp) at Days 1 (baseline), 29, 71, and at weeks 32 and 60. No participants in the RNAseq

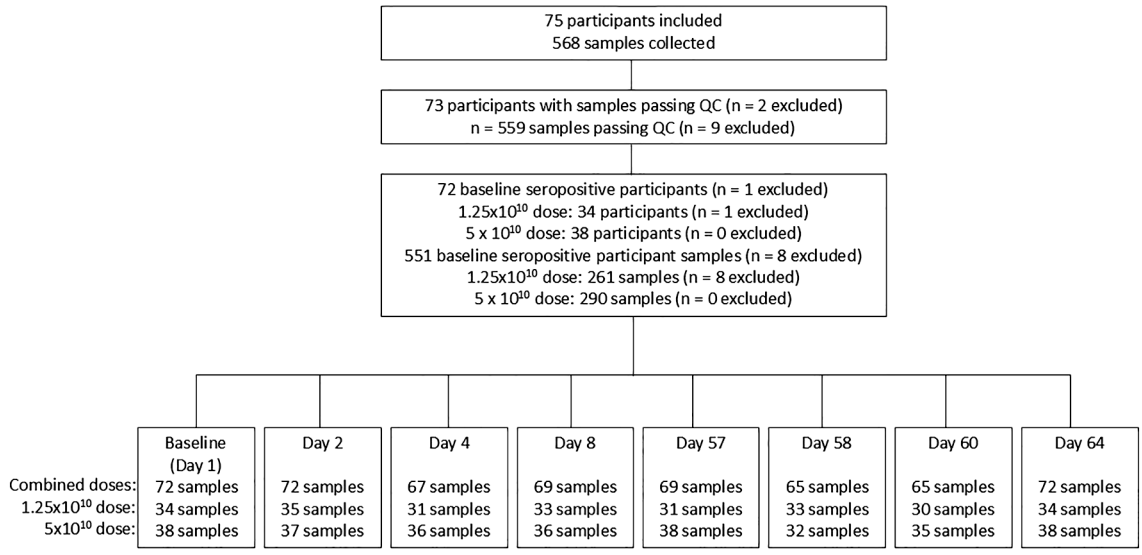

Fig. 1: **Participant and sample flow chart.** Presentation of the number of participants, and corresponding samples, included in the study, the numbers of participants and samples excluded, and the reasons for exclusion. n: number, QC: quality control.

subset had SARS-CoV-2 infections during the course of the study. Most participants were between 18 and 40 years old and male (59.5%), with a median age of 30.5 years and a median body mass index of 25.1 kg/m2. Participants were from Brazil (70.3%), South Africa (16.2%), or the United States (13.5%).

Ad26.COV2.S vaccination elicits stronger spike binding antibody responses post-dose 1 than post-dose 2 in participants with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection

Spike (S)-specific binding antibody levels were similar in the 5×10^{10}vp group and the 1.25×10^{10}vp group, both prior to Vaccination 1 (Day 1) and at post-vaccination time points. S-binding antibody responses peaked 28 days Post-Vaccination 1 (Day 29), with approximately a 5-fold increase from baseline, followed by a slight decline at Day 57 (Fig. 2, Supplementary Table S2). A second dose did not elicit a further increase in S-binding antibody levels, with fold increases from Pre-Dose 2 of 1.1-fold and 0.9-fold in the 5×10^{10}vp group and the 1.25×10^{10}vp group, respectively (Supplementary Table S2). Finally, S-binding antibody responses were durable, with geometric mean concentrations above baseline levels up to approximately 12 months post-vaccination (week 60) (Fig. 2, Supplementary Table S2).

Transcriptional responses induced by Ad26.COV2.S are governed by innate and adaptive immune responses at 1 and 7 days post-vaccination, respectively

Whole blood from participants was collected in PAXgene® RNA tubes Pre-Vaccination 1 and 2 (Days 1 and 57, respectively), on Days 2, 4, and 8 (1, 3, and 7 days Post-Vaccination 1) and on Days 58, 60, and 64 (1, 3, and 7 days Post-Vaccination 2), and was subjected to RNA sequencing. Samples that did not pass quality checks to ensure sufficient quality of the RNA sample were removed from the analysis (Fig. 1). After mapping and filtering genes with low expression, a principal component analysis of the analysed samples indicated that samples from Days 2 and 58 (i.e., 1 Day post-each vaccination), and particularly those from participants vaccinated with the 5×10^{10}vp dose, are more similar to one another than the rest of the samples (Fig. 3A).

The largest number of differentially expressed genes (DEGs; false discovery rate [FDR] p-value < 0.05 and $\log_2$ fold change >0.58) induced by Ad26.COV2.S was observed 1 Day Post-Vaccination, i.e., on days 2 and 58 as compared to the respective pre-vaccination time point (Fig. 3B). The number of DEGs decreased by 3 days Post-Vaccination. Although there was an increase in the number of upregulated DEGs by 7 days Post-Vaccination 1, this was not observed Post-Vaccination 2. No DEGs were found by 56 Days Post-Vaccination 1. Most DEGs were upregulated and, in general, less DEGs and lower fold changes were found Post-Dose 2 than Post-Dose 1 (Fig. 3B, Supplementary Fig. S2).

Gene set variation analysis (GSVA) with BloodGen3 modules revealed that at 1 Day Post-Vaccination, Ad26.COV2.S primarily stimulated innate antiviral and interferon response modules, but other modules including cytokine/chemokine, monocyte, complement, and protein synthesis modules were also induced (Fig. 3C), which include overlapping genes. Lymphocyte and T cell modules were amongst the top down-regulated

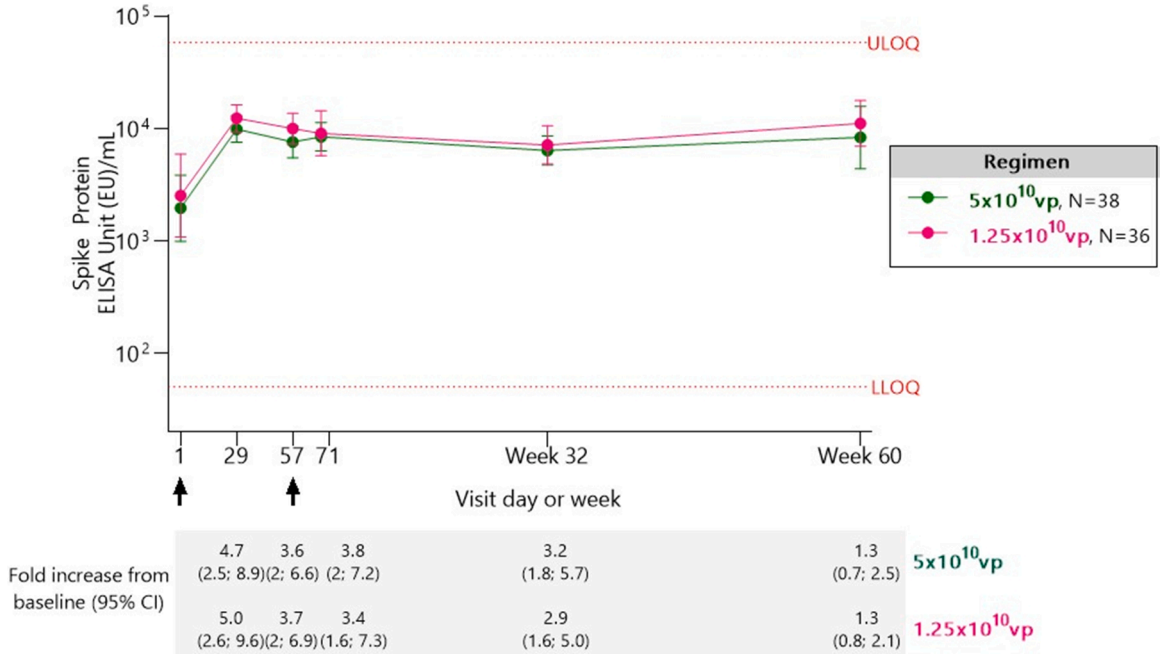

Fig. 2: **Humoural immune responses over time per group.** SARS-CoV-2 Spike binding antibody levels were measured by S-ELISA at Days 1 (Pre-Vaccination 1), 29, 57 (Pre-Vaccination 2), 71, and at weeks 32 and 60. Arrows indicate vaccination with Ad26.COV2.S at Day 1 and at Day 57. The 5 × 10^{10}vp group is shown in green, whereas the 1.25 × 10^{10}vp group is shown in pink. Geometric mean concentrations are shown in the graph as dots with the corresponding 95% CIs. Geometric mean increases, labelled as fold increases from baseline, in each group are summarised below the graph at each Post-Dose 1 time point. N: number of participants with data at baseline. LLOQ: lower limit of quantification, ULOQ: upper limit of quantification.

BTMs (blood transcriptional modules) identified 1 Day Post-Vaccination. Genes composing the top interferon module stimulated (M10.1), such as leucine aminopeptidase 3 (LAP3), interferon regulatory factors, interferon inducible proteins, guanylate-binding proteins (GBPs), signal transducer and activator of transcription 1 and 2 (STAT1/2), tripartite motif containing gene 22 (TRIM22), poly [ADP-ribose] polymerases (PARPs), F-box only protein 6 (FBXO6), and DEXD/H box RNA helicase 60 (DDX60), amongst others, were all strongly upregulated by 1 Day Post-Vaccination 1, with fold changes of up to approximately 7-fold, and their expression decreased at later time points (Fig. 3D). Most DEGs in the T cell module M12.6 were downregulated by 1 Day Post-Vaccination, but fold changes in gene expression were generally low, with up to 0.6-fold change relative to Pre-Vaccination 1.

At 3 Days Post-Vaccination, BTMs with various functions such as interferon, plasma cells, gene transcription, and complement were identified (Fig. 3C). However, at this time point, transcriptional responses were low, with lower statistical significance and BTM score differences than at 1 Day Post-Vaccination. By 7 Days Post-Vaccination, transcriptional responses induced by Ad26.COV2.S were governed by a stimulation of several plasma cell modules, indicating the activation of the adaptive immune response (Fig. 3C). Within the plasma cell module M12.15, all genes such as marginal zone B and B1 cell specific protein (MZB1), joining chain of multimeric IgA and IgM (JCHAIN), CD38 were strongly upregulated, with up to approximately 2.4-fold increase in expression by 1 Day Post-Vaccination 1 (Fig. 3D).

The types of BTMs up- or down-regulated Post-Dose 1 and Post-Dose 2 were similar; however. responses Post-Dose 2 showed smaller score differences and statistical significance (Fig. 3C).

We next evaluated how a plasma cell blood module, previously identified as a universal predictor of vaccine-induced antibody responses,[6,7] was regulated by Ad26.COV2.S. As our analysis used a different BTM database in which the plasma cell blood module M156.1 was not included, we identified the genes composing M156.1[7] and determined their expression over time. All genes, including immunoglobulin genes and B cell regulator genes, except IGHD, were upregulated after Ad26.COV2.S vaccination (Fig. 3E). The expression of these genes was upregulated at 3 Days Post-Vaccination but peaked at 7 Days Post-Vaccination with fold changes of up to 3-fold increase Post-Vaccination 1. However, none of the genes within module M156.1 were correlated with antibody response as assessed by

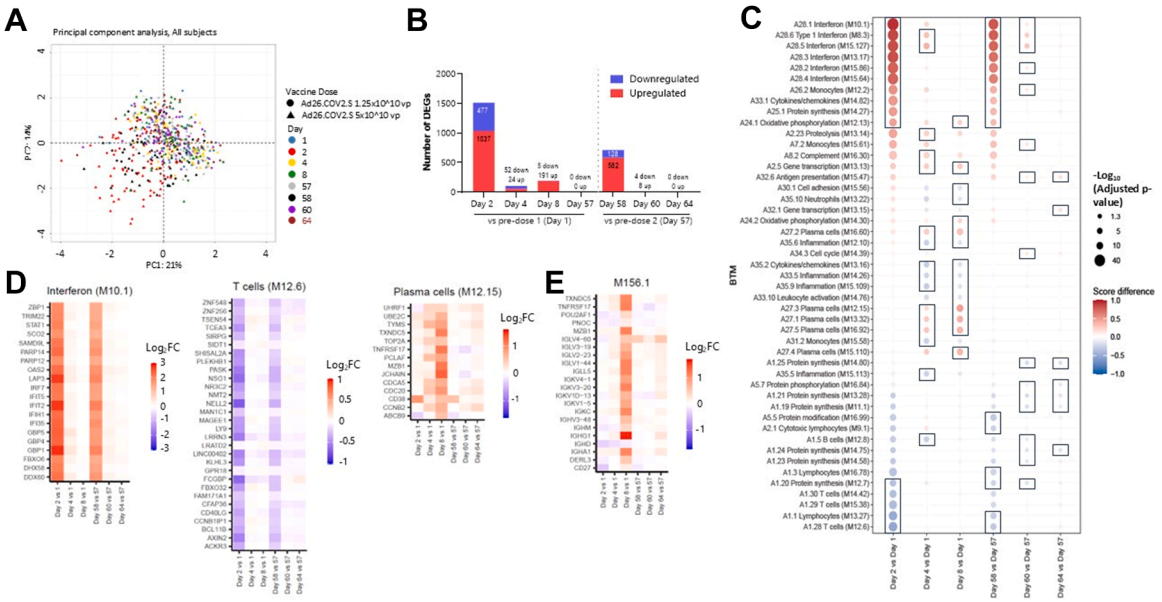

***Fig. 3*: Transcriptional responses post-Ad26.COV2.S vaccination.** (A) Principal Component Analysis of all whole blood samples analysed by RNAseq. The contribution of principal component 1 and 2 to the overall variance are displayed along the x- and y-axes, respectively. Samples from Day 1 through Day 64 are represented by different colours, while the different vaccine dose levels are represented by different symbols. X-linked genes were not excluded from this analysis and no correction was applied. (B) Number of DEGs identified Post-Dose 1 and Post-Dose 2, relative to the respective pre-vaccination time point are shown in or above each bar. DEGs represent those with an FDR-corrected p-value < 0.05 and with a Log_2 fold change >0.58. Pooled dose level results are shown. Blue bars represent the number of down-regulated DEGs while red bars represent the number of upregulated DEGs. (C) Kinetics of selected BTMs identified by GSVA. An overview of the top 10 up- and down-regulated significant BTMs identified at each post-vaccination time point relative to pre-vaccination is shown, along with GSVA score differences and FDR-corrected p-values over time. Results from participants in the 5×10^{10}vp and 1.25×10^{10}vp groups are pooled. Top significant BTMs are defined as those with the highest GSVA score difference and an FDR-corrected p-value < 0.05. Squares indicate the top 10 up- or down-regulated BTMs in each comparison, BTMs for which the description is named 'TBD' are not shown. (D) Log_2 fold changes, relative to the indicated pre-vaccination time point, for individual genes within the Interferon, T cells, and Plasma Cells BTMs. The colouring of the boxes associated with each gene indicates the direction and magnitude of the fold change for each gene, with red end of the spectrum indicating positive log^2 fold-changes, and the blue end of the spectrum indicating negative log_2 fold-changes. (E) Log_2 fold changes, relative to the indicated pre-vaccination time point, for individual genes within the M156.1 module, which was previously identified as a universal predictor of vaccine-induced antibody responses. The colouring of the boxes associated with each gene indicates the direction and magnitude of the fold change for each gene, with red end of the spectrum indicating positive log^2 fold-changes, and the blue end of the spectrum indicating negative log_2 fold-changes. DEGs: differentially expressed genes, BTM: blood transcriptional module. GSVA: Gene Set Variation Analysis.

S-ELISA at 28 days post-vaccination (Supplementary Figs. S3 and S4).

Transcriptional responses are stronger post-dose 1 compared to post-dose 2

We next formally compared transcriptional responses at each corresponding time point Post-Dose 2 versus Post-Dose 1. The largest number of DEGs were found 1 Day Post-Vaccination (Fig. 4A). At all time points, most significant DEGs between Post-Dose 2 and Post-Dose 1 time points and those with the largest fold changes show a negative fold-change, suggesting that the regulation of these transcripts is more elevated Post-Dose 1 as compared to Post-Dose 2 (Figs. 4A and 3B). The identity of the genes in each volcano plot in Fig. 4B can be found in Supplementary Excel File 1.

One Day Post-Vaccination, significant DEGs that showed the largest fold changes Post-Dose 2 compared to Post-Dose 1 included genes involved in the immune response, such as the chemokines CXCL10 (or IP-10), CCL2, and CCL8, and other immune regulators such as major defensin beta 1 (DEFB1), and all had a negative fold-change (Fig. 4C). Three Days Post-Vaccination, DEGs with the largest fold changes Post-Dose 2 compared to post-dose again all showed negative fold-changes and were mostly unannotated or long non-coding RNAs. Seven Days Post-Vaccination, DEGs identified were largely immunoglobulin genes, indicating that the transcription of immunoglobulin-related genes was stronger Post-Dose 1 than Post-Dose 2 (Fig. 4D), consistent with our observations when measuring Spike-specific binding antibody levels

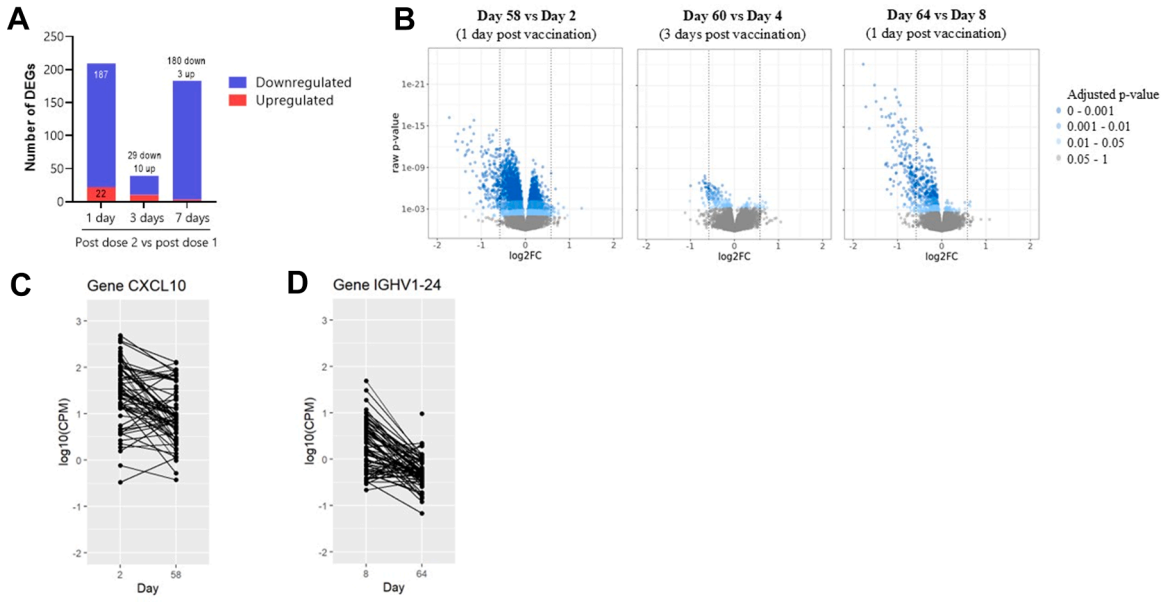

***Fig. 4:* Differential gene expression analysis post-Ad26.COV2.S vaccination Post-Dose 2 versus Post-Dose 1.** Pooled dose level results are shown. (A) Number of DEGs identified Post-Dose 2 versus Post-Dose 1, at each indicated post-vaccination time point (1 Day, 3 Days and 7 Days Post-Vaccination). DEGs represent those with an FDR-corrected p-value < 0.05 and with a $\log_2$ fold change >0.58. Blue bars represent the number of down-regulated DEGs while red bars represent the number of upregulated DEGs. (B) Volcano plots display the DEGs identified following a second Ad26.COV2.S vaccination relative to a first Ad26.COV2.S vaccination, at each indicated post-vaccination time point. Log_2 fold changes are shown on the x-axis, and raw p-values on the y-axis. (C) Transcript levels measured 1 Day Post-Dose 1 and Post-Dose 2. CXCL-10 is shown as a representative DEG identified at 1 Day post-each vaccination. (D) Transcript levels measured 7 days Post-Dose 1 and Post-Dose 2. IGHV1-24 is shown as a representative DEG identified at 7 Days post-each vaccination. CPM: counts per million.

induced by Ad26.COV2.S Post-Dose 1 and Post-Dose 2 (Fig. 2).

Ad26.COV2.S vaccination with different dose levels has minimal effect on transcriptional responses

Finally, we compared transcriptional responses between dose levels at each time point. Vaccination with Ad26.COV2.S at 5×10^{10}vp as compared to 1.25×10^{10}vp did not result in differential gene expression at any time point except Day 2 (1 Day Post-Vaccination 1) (Figs. 5A and 4B). At all other post-vaccination time points, differential gene expression was similar to that at baseline (Day 1), with a maximum of one DEG identified. At Day 2, a total of 392 genes showed higher expression and 945 genes showed lower expression (Fig. 5B) in the 5×10^{10}vp group as compared to the 1.25×10^{10}vp group. Of these, 63 and 289 (total 352) genes with lower and higher expression, respectively, showed a $\log_2$ fold difference higher than 0.58 (Fig. 5A). The identity of the genes in each volcano plot in Fig. 5B can be found in Supplementary Excel File 2.

DEGs that were more highly expressed in the 5×10^{10}vp group as compared to the 1.25×10^{10}vp group at Day 2 consisted of genes associated with antiviral, cytokine, and innate interferon responses, including lysosome-associated membrane glycoprotein (LAMP3), interferon alpha inducible protein 27 (IFI27), and chemokine ligand 17 (CXCL17), as well as long non-coding RNAs (Figs. 5B and 4C). Genes with significantly lower expression (e.g., oligodendrocyte transcription factor 2 (OLIG2) and RAP1 GTPase activating protein (RAP1-GAP) were biologically unlinked, making it difficult to determine whether their differential expression might have any biological significance.

When each group (5×10^{10}vp or 1.25×10^{10}vp dose level) was compared to baseline, DEGs were regulated similarly (either up- or down-regulated) in both groups, indicating that the differences between dose levels are attributed to differences in magnitude of expression rather than function of the transcriptome response (Fig. 5C). Further, in comparison with DEGs which were upregulated compared to baseline, those that were down-regulated compared to baseline showed smaller differences between the 5×10^{10}vp and the 1.25×10^{10}vp group (Fig. 5C).

These findings indicate that there are no disparities in the nature of DEGs following Ad26.COV2.S vaccination at the 5×10^{10}vp and 1.25×10^{10}vp dose levels, however the higher dose level appears to elicit slightly elevated transcriptional responses.

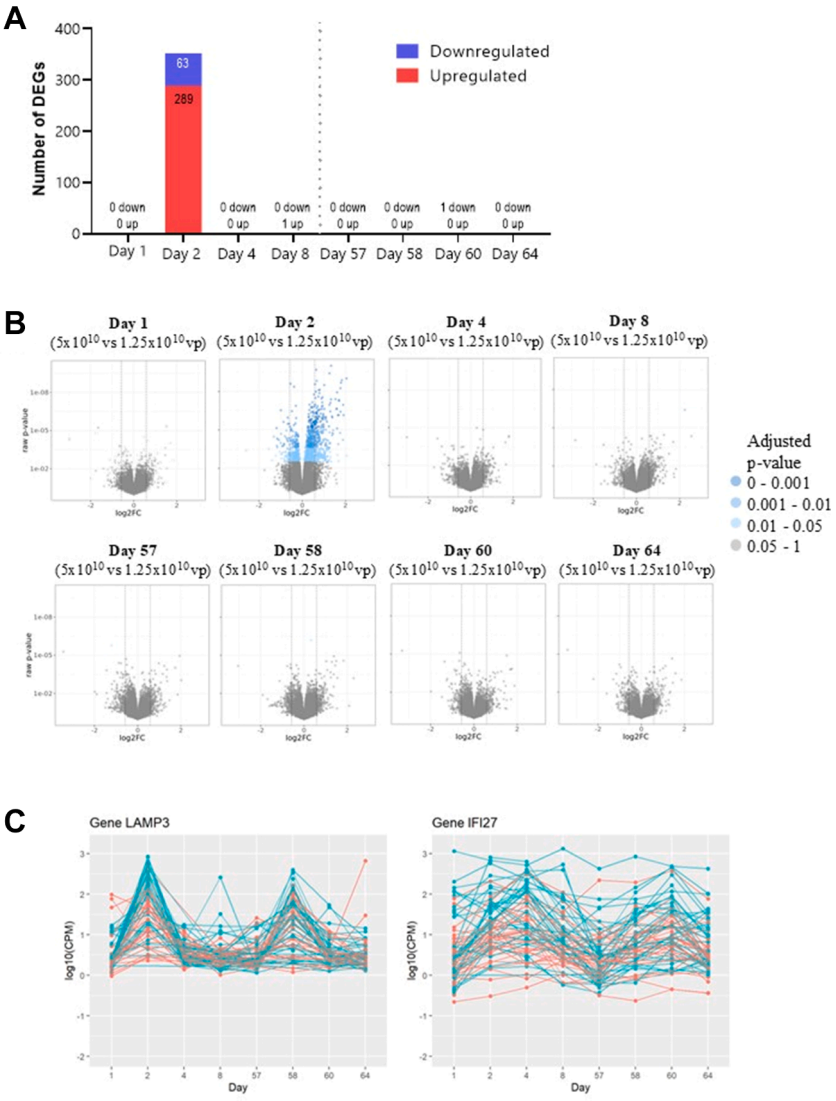

***Fig. 5*: Differential gene expression analysis following Ad26.COV2.S vaccination at the 5 x 10^{10}vp dose level versus 1.25 x 10^{10}vp dose level.** (A) Number of DEGs identified in the 5 × 10^{10}vp group relative to the 1.25 × 10^{10}vp group, at each indicated time point. DEGs represent those with an FDR-corrected p-value < 0.05 and with a $\log_2$ fold change >0.58. Blue bars represent the number of down-regulated DEGs while red bars represent the number of upregulated DEGs. (B) Volcano plots display the DEGs identified in the 5 × 10^{10}vp group relative to the 1.25 × 10^{10}vp group, at each indicated time point. Log_2 fold changes are shown on the x-axis, and raw p-values on the y-axis. (C) Transcript levels of selected DEGs: LAMP3, IFI27, OLIG2, and RAP1GAP. CPM: counts per million.

Discussion

In this study we used RNA sequencing to profile Ad26.COV2.S-induced transcriptional responses in individuals who were previously unvaccinated against COVID-19 but who had pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection. Our data show that Ad26.COV2.S upregulated the expression of genes related to both innate and adaptive immune responses in whole blood. The up-regulation of these genes occurred at different timings, with increased expression of innate immune response genes at 1 Day Post-Vaccination while adaptive immune response genes exhibiting up-regulation from 7 Days Post-Vaccination. These findings align with previous studies investigating transcriptional responses following COVID-19 vaccination(s) against other

unrelated pathogens.[6,9,11] Furthermore, our results show that transcriptional responses were similar across different dose levels of the vaccine.

Consistent with other studies, we observed that differentially regulated blood transcriptional modules 1 Day Post-Vaccination were associated with antiviral, interferon, cytokine, and monocyte responses. This suggests that the innate immune system is activated as early as one day after Ad26.COV2.S vaccination, which corresponds to the timing of onset of systemic reactogenicity.[15] Within these modules, transcripts such as leucine aminopeptidase 3 (LAP3), leptin, signal transducer and activator of transcription 1 and 2 (STAT1/2), tripartite motif containing genes (TRIMs), interferon regulatory factors (IRFs), interferon induced proteins (IFIs), and interferon gamma inducible genes were upregulated after vaccination. Notably, LAP3 was highly upregulated on Day 2. LAP3 is known to exhibit antiviral activity as it was identified via its ability to restrict HIV replication.[16] Previous independent studies investigating transcriptional responses induced by an adenovirus serotype 26-based Ebola virus vaccine[4] and evaluating biomarkers following adenovirus infections[17] have also shown up-regulation of LAP3, suggesting that the induction of LAP3 is a specific marker induced by Adenovirus-based vaccines.

At 3 Days Post-Vaccination, we observed little transcriptional activity, which may be due to reduced gene expression regulation overall or a lower number of vaccine-induced immune cells circulating in whole blood at this time point. The type of differentially regulated genes and blood transcriptional modules were associated with several processes, including proteolysis, gene transcription, as well as inflammation, complement and interferon responses, suggesting that innate immunity is still transcriptionally active at three days post-Ad26.COV2.S vaccination. By 7 Days Post-First Vaccination, the transcriptional response was dominated by an increase in immunoglobulin gene expression indicating a shift towards humoural and B cell responses. These observations suggest that the activation of the adaptive immune response is already detectable in whole blood by seven days after the first dose of Ad26.COV2.S and that, in previously SARS-CoV-2 exposed individuals, memory B cells are proliferating or differentiating not only in lymph nodes after antigen re-exposure, but also in the blood. This may be different in naïve individuals where most of the B cell activities after antigen exposure occurs in secondary lymphoid organs. It is worth noting that while B cell responses are prominent, transcriptional signatures of T cell activation were not robustly detected in this dataset. As the Ad26.COV2.S vaccine did induce robust CD4 and CD8 T cell responses in previous studies,[15,18] the lack of a T cell transcriptional signal in this study may be due to the strong B cell signature observed at Day 8, resulting in proportionally higher B cells and effectively masking T cell transcriptional responses. It is also worth noting that a recent study proposed the expression of a plasma cell blood module (M156.1) as a universal predictor of vaccine-induced antibody responses across multiple vaccines.[6,7] Although the study employed a different set of BTMs compared to those used in this study, genes included in this module, such as immunoglobulin genes (IGKV1-5, IGLV4-60, IGKC, IGHD, IGHM) and B cell regulator genes (TNFRS17, POU2AF1, MZB1, CD27) were upregulated 7 days after Ad26.COV2.S vaccination, confirming the commonality of such signatures induced by vaccines. Despite the previous finding that module M156.1 is a universal predictor of vaccine-induced antibody response for other vaccines, in this study none of the genes within module M156.1 were correlated with antibody response as assessed by S-ELISA at 28 days post-vaccination. Another recent study revealed that 7 days post-vaccination with an H5N1 influenza vaccine, platelet and cell-adhesion-related module M196 predicted antibody response durability.[19] While we identified seven erythroid or platelet BTM modules that were upregulated upon vaccination in our GSVA analysis, only one erythroid module (BloodGen3 Module M14.68) was ranked among the top 100 modules. None of the erythroid and platelet genes identified in our analyses matched with the genes found in the platelet activation/actin binding module M196 (data not shown).

Interestingly, our findings revealed that transcriptional responses following Ad26.COV2.S vaccination were, overall, stronger after the first dose compared to the second dose. These results were consistent with the observed humoural immune responses, since a second dose of Ad26.COV2.S did not elicit a further increase in S-binding antibody levels in the studied population who had pre-existing immunity to SARS-CoV-2 due to prior SARS-CoV-2 infection. This is in agreement with a published study in adolescents, which showed that Ad26.COV2.S vaccination elicited a stronger immune response Post-Dose 2 in participants who were SARS-CoV-2 seronegative versus seropositive at baseline, and that SARS-CoV-2 binding antibody levels prior to vaccination negatively correlate with fold increases post-vaccination.[20] This is unlikely to be due to the induction of antibodies directed against the Ad26 vector, as a second dose of Ad26.COV2.S at 5×10^{10}vp, administered with the same interval as in this study, was previously demonstrated to elicit a stronger binding antibody response than the first dose in adults without pre-existing immunity.[21] Although the Ad26.COV2.S study in adults without pre-existing immunity[21] did not examine the transcriptional response, studies using mRNA-based COVID-19 vaccines in adults without pre-existing immunity have shown greater transcriptional responses after the second vaccination compared to the first.[10,11] A similar observation of a stronger transcriptional response after dose 2 was previously observed in

a study of transcriptional responses after hepatitis B vaccination with two doses of either Engerix-B™, Fendrix™, one of three different formulations of GSK223192A, or a booster dose consisting of hepatitis B surface antigens.[22] The difference between our results and those of other studies with different COVID-19 vaccines can likely be attributed to the SARS-CoV-2 exposure history of the populations examined in those studies. While our analysis focused on a population with pre-existing SARS-CoV-2 immunity due to prior SARS-CoV-2 infection, the published studies were conducted on individuals without previous exposure to the virus. It may be that there is a mechanism which limits the total level of antibodies or plasmablasts present in the blood and which, therefore, prevents the upregulation of relevant genes after the second dose. However, whether there is a difference in the transcriptional response Post-Dose 2 versus Post-Dose 1 in SARS-CoV-2 naïve and pre-exposed individuals remains to be determined.

It has been recently demonstrated that pre-existing antibody titres against SARS-CoV-2 may limit the amount of antigen available to prime B cell responses post-mRNA vaccination, leading to lower fold increases in humoural immune responses particularly in individuals with higher pre-vaccination titres.[23] In line with this, we observed strong up-regulation of immunoglobulin-related genes 7 days after the first dose, which was not observed after the second dose, when Spike-binding antibody levels were higher prior to Ad26.COV2.S vaccination. Therefore, the absence of significant DEGs or BTMs related to antibody or B cell responses after the second Ad26.COV2.S vaccination may be explained by the presence of high antibody titres prior to the second vaccination, particularly in a population with pre-existing SARS-CoV-2 immunity for which robust Post-Dose 1 antibody responses are expected.

Our study should be interpreted within the context of certain limitations. First, the absence of a placebo group in the COV3003 clinical study limits the ability to make direct comparisons with a control group. However, we were able to evaluate transcriptional responses induced by Ad26.COV2.S by analysing samples collected prior to vaccination. Secondly, our study specifically included individuals who were SARS-CoV-2 seropositive at baseline. Therefore, the transcriptional responses described herein represent primarily a population with pre-existing immunity to SARS-CoV-2 due to prior SARS-CoV-2 infection. This is particularly relevant in the current epidemiological context, where a majority of the population has been previously exposed to the virus. It remains to be determined if similar responses would be observed in a population that reflects the earlier stages of the COVID-19 pandemic without pre-existing SARS-CoV-2 immunity. Finally, a key limitation of whole-blood transcriptomics is that transcriptional differences may be driven by shifts in cellular composition, which can impact the results and conclusions of whole-blood transcriptomics studies.

In conclusion, antiviral, interferon, cytokine, and monocyte blood transcriptional modules were differentially regulated one day post-Ad26.COV2.S vaccination, which is consistent with observations in other vaccine transcriptomics studies. Innate immunity was still transcriptionally active at three days post-vaccination, at lower levels than one day post-vaccination. Increased immunoglobulin gene expression dominated the transcriptome response by seven days post-vaccination, indicating a shift towards humoural and B cell responses and activation of adaptive immune responses. In general, transcriptional responses post-Ad26.COV2.S appear stronger after the first dose versus the second dose. Our findings add to the limited data on innate immune responses to vaccination with adenovirus-based vaccines in a clinical setting and contribute to increasing our understanding of the mechanisms behind the development of innate and adaptive immune responses that may contribute to protective immunity.

Contributors

Author contributions are as follows, as defined by the CrediT author contributions policy:

Conceptualisation (ideas; formulation or evolution of overarching research goals and aims): VR, JT, NH, JS, MLG, FS, JH, JRG, CM; Methodology (development or design of methodology; creation of models): JT, JS; Software (programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components): JT, JS; Validation (aerification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs): VR, JT, NH, JS, CM; Formal Analysis (application of statistical, mathematical, computational, or other formal techniques to analyse or synthesise study data): JT; Resources (provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools): VR, JT, JS, FS, JRG; Data Curation (management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse): VR, JT, NH, JS, CM; Writing (preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation))—Original Draft: VR, JT, CM; Writing – Reviewing and Editing (preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision—including pre-or postpublication stages): VR, JT, NH, JS, MLG, FS, JH, JRG, CM; Visualisation (preparation, creation and/or presentation of the published work, specifically visualisation/data presentation): VR, JT, CM; Supervision (oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team): JS, JH, CM; Project Administration (Management and coordination responsibility for the research activity planning and execution): VR, NH; Verification of the underlying data: VR, JT, CM.

All authors have read and approved the final version of the manuscript.

Data sharing statement

The raw data, including RNA sequences, supporting the conclusions of this article will be made available by the authors, without undue

reservation. Janssen has an agreement with the Yale Open Data Access (YODA) Project to serve as the independent review panel for the evaluation of requests for clinical study reports and participant-level data from investigators and physicians for scientific research that will advance medical knowledge and public health. Data will be made available following publication and approval by YODA of any formal requests with a defined analysis plan. Review of requests is expected to be completed within 30 days. Depending on the data requested, data will be made available either via a password-protected personalised account on a secure data sharing platform or via the Yale Secure File Transfer Service. For more information on this process or to make a request, please visit the Yoda Project site at http://yoda.yale.edu. The data sharing policy of Johnson & Johnson Innovative Medicine is available at https://www.jnj.com/innovativemedicine/our-innovation/clinical-trials/transparency.

Declaration of interests

All authors were employees of Johnson & Johnson Innovative Medicine at the time this work was completed and may hold Johnson & Johnson stocks or stock options. Johnson & Johnson Innovative Medicine exclusively funded the study and was involved in the design, collection, analysis, and interpretation of data and the decision to submit the manuscript for publication. V.R., M.L.G, F.S., J.H, J.R.G, and C.M. received support for attending conferences and travel from Johnson & Johnson Innovative Medicine. V.R. and F.S. are current employees of Glaxosmithkline and may hold company stocks or stock options. F.S. is an author on active patent US12233118B2. V.R. received support for attending conferences and travel from Glaxosmithkline and is an author on pending patent US20230174953A1. J.R.G. is a current employee and member of the safety review board at Vicebio.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2026.106270.

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