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Rapid diagnostic test for assessment of cellular immune response in a viral infection as a model

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IGRA
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A B S T R A C T

The global SARS-CoV-2 pandemic has highlighted the critical importance of evaluating adaptive immunological status in both infected individuals and those who have been vaccinated. While the humoral immune response has been extensively studied, the cellular immune component has not progressed as rapidly due to challenges in developing rapid diagnostic tests for activated T cells. This study introduces a rapid, user-friendly assay aimed at addressing this need by using the early expression of interferon-gamma (IFN-γ) transcripts for rapid and direct evaluation. Using a novel design, this assay measures IFN-γ mRNA rather than its protein levels, thereby significantly shortening the time to result compared to traditional methods. The process involves incubating whole blood with a mixture of Spike and Nucleocapsid peptides, followed by immunomagnetic separation of CD3+ T cells and subsequent double-tagging RT-PCR. A lateral flow assay (LFA) is then used for detection, allowing IFN-γ mRNA levels to be assessed rapidly. Non-stimulated samples are used as controls to establish baseline IFN-γ expression. The intensity of the LFA signals from the stimulated samples is then compared to these baseline values using a smartphone interface. Furthermore, the study includes a time course analysis to monitor the T cell response dynamics over time in infected individuals. This LFA-IFN-γ represents a significant progress in the field of rapid diagnostics, offering a one-day protocol that simplifies testing procedures and is especially suitable for low-resource settings.

1. Introduction

The increasing significance of disease outbreaks in health care accentuate the need of their early diagnosis and the monitoring of the evolution of infected patients. The immune system has evolved to provide protection to multiple pathogens by using different effector mechanisms. The final goal is to eliminate the pathogen and generate immunological memory through the activation of pathogen-specific B and T lymphocytes. B cells lead the humoral immune response secreting pathogen-specific antibodies that help neutralizing and opsonizing the target [1]. On the other hand, cellular immune response is based on the activation of pathogen specific CD3+ T cells [2], including CD8+ T cells or cytotoxic T cells responsible for the recognition and lysis of target-infected cells, and CD4+ T cells critical due to their capacity to orchestrate the immune response and to their implication in B-cell activation for antibody production [3,4]. Antigen-specific T cells become activated when they detect peptides from pathogens that are bound to molecules known as Major Histocompatibility Complex (MHC) molecules generating peptide-MHC (pMHC) complexes. This process is facilitated by professional antigen-presenting cells or infected cells that express pMHC complexes exposed on the membrane to be recognized by T cells through their antigen-specific receptors [5]. One of the consequences of T-cell activation relies on the production and release of soluble mediators as cytokines. For the activation of cytotoxic T cells, the release of interferon-gamma (IFN-γ) is crucial [6,7]. It is defined as a pro-inflammatory cytokine belonging to the interferon family. In

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addition to cytotoxic T cells, IFN-γ is also produced by activated Th1 cells (a subtype of Th cells), NK cells, and NKT cells [8].

The determination of cytokine production as indirect evidence of T-cell activation by specific antigens was performed for the first time by the so-called IGRA test (interferon gamma release assay) for the diagnosis of latent tuberculosis infection [9]. Different methodological approaches have been developed for the measurement of T-cell responses against *Mycobacterium tuberculosis* based on the detection of IFN-γ production upon specific stimulation. There are two currently approved by the FDA, QuantiFERON®-TB Gold Plus (QFT-Plus, FDA P010033) and T-SPOT®.*TB* test (T-Spot, FDA P070006). Briefly, the QFT-Plus test is an ELISA-based method that quantifies IFN-γ released into the plasma following an overnight incubation of whole blood with *Mycobacterium tuberculosis* derived immunogenic peptides. In contrast, T-Spot quantifies the IFN-γ producing cells, by requiring the prior isolation and overnight stimulation of peripheral blood mononuclear cells (PBMCs). The kits for diagnosing latent tuberculosis infection have led to the creation of similar assays for detecting other pathogens, including SARS-CoV-2 [10–12]. Other alternative assay is the determination of IFN-γ by intracellular flow cytometry in CoVITEST [13,14], or through the IFN-γ transcript amplification followed by electrochemical genosensing, as previously reported by our group [15]. One of the crucial features to set up an IGRA test is the previous knowledge of the pathogen-immunogenic peptides. The evaluation of the cellular immune response provides insights into the quality and durability of immunity during and after infection, as well as following vaccination [16–18].

The global pandemic triggered by the SARS-CoV-2 coronavirus highlighted the importance of evaluating the immunological status of populations. At-home rapid tests primarily focused on detecting the presence of the virus, without assessing the immune response of infected individuals. Although the humoral response to SARS-CoV-2 was extensively analysed through the first *in vitro* diagnostic tests in hospitals [19], assessing the cellular response proved challenging. This difficulty derived from the requirements to process PBMCs and the initial absence of identified immunogenic targets at the outbreak onset. Several of the most severe COVID-19 symptoms were linked to immune response dysfunction [20,21]. The capability to classify patients based on their prognosis could have been crucial in preventing the collapse suffered at hospital emergency services. Therefore, the need for a rapid, user-friendly test to detect activated specific T cells became a significant priority.

The current study illustrates an improvement to the original IGRA design through the integration in the test of antiCD3 and oligo(dT)-modified magnetic particles [22]. This approach streamlines the process by targeting IFN-γ mRNA, allowing for a shorter peptide incubation in whole blood compared to the traditional protein-based method. Furthermore, the use of antiCD3-modified magnetic particles selectively targets IFN-γ producing T-cells, improving the efficiency of the test. Sensitivity is also increased by capturing, amplifying, and detecting IFN-γ mRNA. The detection of IFN-γ amplicons is efficiently performed by a lateral flow assay (LFA), referred to as LFA-IFN-γ, with visual readout, reducing the need for instrumentation.

This study also compares the performance of commercially available gold nanoparticles (AuNPs), the established standard in LFA technology, with in-house avidin-modified carbon nanoparticles (CNPs) proposed as cost-effective and sensitive alternatives [23]. Previous studies suggest that CNPs may offer enhanced visual contrast, potentially leading to improved assay sensitivity [23]. The aim is to assess the viability of CNPs against AuNPs as an economical diagnostic tool suitable for global health contexts using the new *in vitro* diagnostic test, LFA-IFN-γ, and SARS-CoV-2 infection as a model.

2. Methods

2.1. Rapid diagnostic test for assessing and monitoring cellular immune response

All the solutions, reagents and equipment are detailed listed on Supp. data, S1. The procedure from blood stimulation to visual readout based on a paper-based immunochromatography test, the LFA-IFN-γ, is schematically described in Fig. 1.

2.2. Whole blood stimulation, immunomagnetic separation of the lymphocytes and capturing of the transcripts

The assay was tested using heparinized whole blood from SARS-CoV-2 infected patients 10–45 days after receiving a negative antigen test result, as well as from non-infected donors. Each 3 mL sample was processed under three different conditions (1 mL each), as shown in Fig. 1, panel A1: i) negative control (non-stimulated blood) to assess the basal condition; ii) positive control, stimulated with phytohemagglutinin (PHA, 5 μg mL^{-1}) to induce non-specific mitogenic stimulation in human lymphocytes; and iii) the test sample, stimulated with peptides from the two main SARS-CoV-2 antigens, spike and nucleocapsid proteins (S and N peptides, 2.5 μg mL^{-1} each). Each sample was incubated at 37°C for 4 h.

Next, the immunomagnetic separation of T lymphocytes were performed, as shown in Fig. 1, panel A2, by incubation directly with $1 \cdot 10^7$ antiCD3-MPs for 30 min with rotation at 4°C (Fig. 1, panel A2). CD3+ T cells were then washed twice with isolation buffer. Isolated CD3+ T cells were resuspended with 1 mL of Lysis/Binding buffer and disrupted using a 21 G needle to release the genetic material (Fig. 1, panel A3). The antiCD3-MP were discarded, and the supernatant was kept for further mRNA isolation. In all instances, after incubation or washing step, a magnetic separator was positioned under the tubes until pellet formation on the tube side wall, followed by supernatant separation.

The mRNA capture was carried out with oligo(dT)-MP following the manufacturer protocol. Briefly, 15 μL of oligo(dT)-MP were added into the supernatant and the mixture was incubated 5 min at 750 rpm at RT. mRNAs attached to oligo(dT)MP were washed with 500 μL of washing buffer A and then 500 μL of washing buffer B and with 100 μL of RNase-free water (Fig. 1, panel A4). Finally, the MPs were resuspended in 12 μL RNase-free water to perform the cDNA retrotranscription protocol.

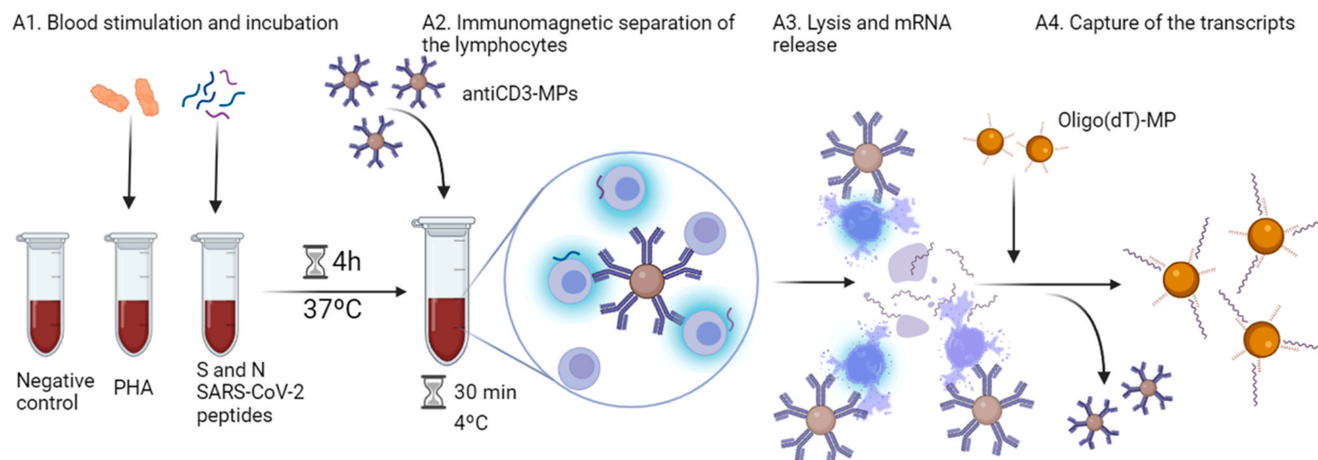
2.3. Double-tagging RT-PCR on magnetic beads

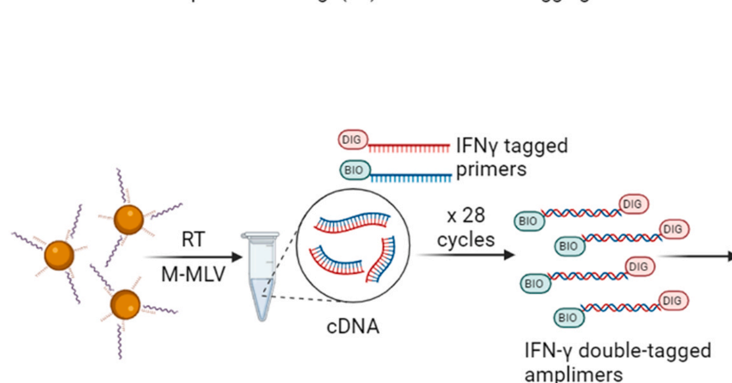
dNTPs were added to the mRNA attached to oligo(dT)-MP and incubated 5 min at 65°C. Then the master mix was added and incubated 2 minutes at 37°C. The M-MLV retrotranscriptase was finally added at 200 U to the mixture with final volume of 20 μL (Fig. 1, panel B1). Reverse transcription was performed for 50 min at 37°C, 15 min at 70°C.

Double-tagging PCR was performed to amplify IFN-γ cDNA using double-tagged Digoxigenin/Biotin-labelled primers. The PCR was performed in 15 μL of reaction mixture per sample. Master mix was added to 2 μL of the cDNA. Amplification was performed after incubation 2 min at 94°C with 28 cycles (94°C for 15 s, 60°C for 15 s, 68°C for 15 s) and analysed using 2 % agarose gel electrophoresis in TAE buffer 1× Syber Safe dye, as well as by LFA.

2.4. Lateral flow assay

The strips for the LFA were prepared by dispensing the antibody anti-digoxigenin (0.5 mg mL^{-1}) as a test line and BSA-biotin (1 mg mL^{-1}) as control line onto a nitrocellulose membrane with an Isoflow reagent dispensing system. In-house avidin-modified carbon nanoparticles (CNP) prepared as described in S2, Supp. data, or commercial streptavidin-modified gold nanoparticles (AuNP) were diluted 1/5 with





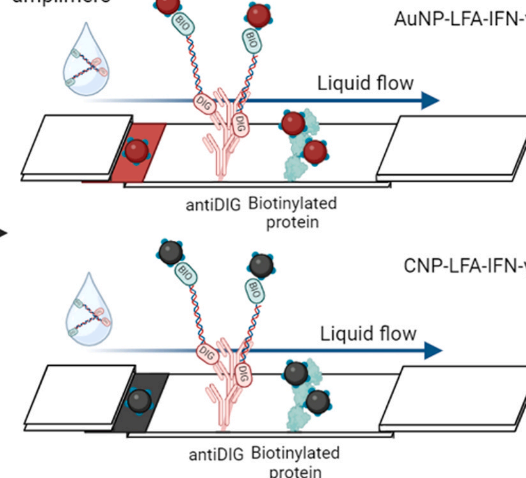


Fig. 1. Schematic representation of the LFA-IFN-γ rapid diagnostic test for cellular immune response. A. Whole blood stimulation, immunomagnetic separation and capture of the transcripts. A1. Whole blood samples are incubated with SARS-CoV-2 peptides (S and N) or PHA (phytohemagglutinin). A negative control without stimulants is also processed, leaving a tube unstimulated (IFN- γ basal level). A2. Immunomagnetic separation of lymphocytes, using antiCD3 magnetic particles (antiCD3-MPs). A3. Lysis and mRNA release. Lysing buffer is added to the isolated lymphocytes, causing cell disruption and release of mRNA. A4. Transcripts capture. Released mRNA is captured using oligo(dT)-coated magnetic particles (oligo(dT)-MP) to isolate the mRNA. B. Double-tagging RT-PCR and Lateral Flow Assay. B1. Reverse transcription and double-tagging PCR. The mRNA is reverse transcribed into cDNA. Following reverse transcription, a double-tagging PCR amplifies IFN-γ specific sequences using IFN-γ tagged primers (with biotin and digoxigenin). B2. Lateral flow assay with CNP and AuNP for detection of IFN-γ amplicons. The LFA strips contain a test line with anti-digoxigenin and a control line with biotinylated protein. For detection, two types of nanoparticles are used: in-house avidin-modified carbon nanoparticles (CNP) and commercial streptavidin-modified gold nanoparticles (AuNP), which bind to the biotin and digoxigenin tags on the amplicons, allowing visualization of the results as the sample flows across the strip. Created with Biorender.com.

conjugation buffer and 25 μL of the mixture were dropped on the conjugate pad and air dried. Both components as well as the sample and the absorbent pads were deposited into a 5 mm adhesive backing pad as well as the previously cut sample pad and absorbent pad (LFA depicted in Fig S3.1 Supp. Data).

On to the sample pad, 5 μL of the PCR product (double-tagged IFN-γ amplimers) diluted with 20 μL of running buffer were dropped and followed by 200 μL of running buffer (Fig. 1, panel B2). The result was registered after 15 min of the buffer deposition through a photograph, taken with a smartphone. To ensure consistent image quality and eliminate luminosity-related biases, all pictures were taken under the same conditions. This process involved using a portable photographic studio equipped with 1100 lm LED lights maintaining a colour temperature of 6000–6500 K and the picture taken with a smartphone camera (12 megapixels). Then, the pictures were analysed using the ImageJ gel analysis toolbox. The results were obtained by analysing the area under the curve of the test lines. Any LFA lacking a visible control line was invalidated.

2.5. Study design

This is a prospective observational and control study that includes eleven individuals. This cohort was divided in individuals that were vaccinated but presumably not infected with SARS-CoV-2 virus (n=5), and individuals that were vaccinated and infected (n=6). Infection with SARS-CoV-2 was confirmed using an antigen test, and blood samples were collected after the test results turned negative. The individuals infected exhibited mild disease symptoms, lasting less than two weeks. Demographic information of the study is collected in Table S4 Supp. data. The longitudinal study was conducted with the infected individuals recruited the first month after the infection. Blood was drawn at the Autonomous University of Barcelona Health Care Service and

informed consent was signed by all volunteers included in the study.

All works were performed in a biosafety cabinet, and all material was decontaminated by autoclaving or disinfected before discarding following US Department of Health and Human Services guidelines for level 2 laboratory Biosafety.

2.6. Statistical analysis

The statistical analyses and calculations were performed using Prism v 10.0.1 (GraphPad, San Diego, USA). The calibration curves were adjusted using a non-linear regression model, specifically the log(inhibitor) vs. response curve with a three-parameter logistic model. The limit of detection was calculated based on the values of negative controls for each curve, using a one-tailed test (Student's t-test, $p < 0.05$). A one-way ANOVA with multiple comparisons was performed to determine statistical differences between the different conditions included in the study, with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Rapid diagnostic test for assessing cellular immune response

The optimization of the lateral flow, including the readout system based on CNP and the analytical performance, is discussed in S3 (Supp data). Two detection systems were tested in this study in parallel: CNP, as a cost-effective and easily prepared readout system, and AuNP, as a gold-standard method, to compare their suitability for the sample evaluation (as outlined in Fig S3.1, Supp data). As the CNPs were prepared in-house, the stability of the avidin-modified CNPs after their integration into the LFA strip was evaluated over a period of eight weeks. The results are shown in Fig. S3.2, Supp. data. The limit of detection (LOD) for the LFA was determined to be 695 cells for AuNP and 1929 cells for CNP using smartphone readout, while the LOD for visual readout was identified as 10,000 cells for both signal-generating systems (as shown in Fig. S3.3, Supp. data). Whole blood samples were

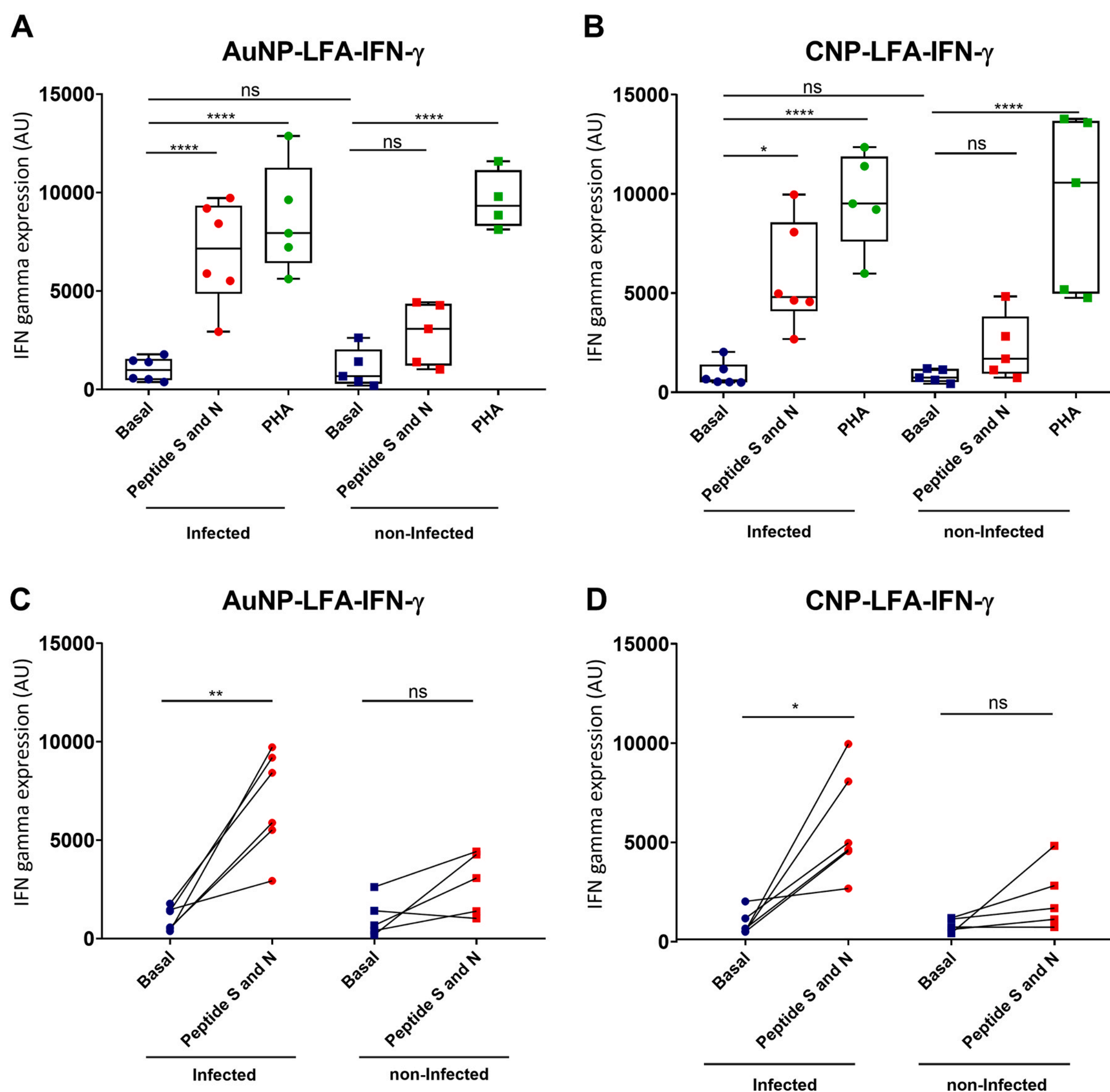


Fig. 2. Box and whisker plots for IFN-γ production between infected and non-infected individuals using AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ. The signal obtained with the test line (and displayed in Fig. S5.1, Supp. data) was treated by ImageJ software and values were represented for the Infected group (circles) and the non-Infected (squares). Whole blood samples were incubated for 4 h in different conditions: non-stimulated (in blue); stimulated with S and N peptides (in red); and stimulated with PHA (in green). Panels A and C and panels B and D show the data from AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ, respectively. The statistical analysis was performed by a multiparametric ANOVA (panels A and B) and Student's *t*-test, at the 95 % significance level (panels C and D), with symbols indicating levels of statistical significance: 'ns' for $P > 0.05$ (not significant), '*' for $P \leq 0.05$, '**' for $P \leq 0.01$, '***' for $P \leq 0.001$, and '****' for $P \leq 0.0001$, and the results, shown in Table S6.1 and S6.2, Supp data, respectively.

analyzed using both readout methods, referred to for simplicity as AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ, based on AuNP and CNP as the signal-generating systems, respectively. Two groups of vaccinated individuals were examined i.e., individuals which, presumably, have not been previously infected with SARS-CoV-2 and individuals recently infected (Supp. data Table S4). For each individual, 1 mL whole blood was processed for each stimulation condition, as schematically described in Fig. 1.

The results outlined in Figure S5.1, Supp. data, are summarized in Fig. 2, illustrates the detection of IFN-γ production in both infected and non-infected individuals, using two LFA detection methods: AuNP-LFA-IFN-γ (Panels A and C) and CNP-LFA-IFN-γ (Panels B and D). As depicted in panels A and B of Fig. 2, the LFA tests, whether AuNP or CNP-based, successfully detected activated T-cells in infected individuals after only a 4-h incubation. This rapid detection capability is a standout feature of this tests, distinguishing them from other commercial assays, summarized in Table S7 (Supp. Data).

For the infected group, there was a significantly higher IFN-γ expression when blood was specifically stimulated with both peptides S and N, compared to the basal (non-stimulated) condition. This was expected due to the active infection prompting an immune response, characterized by T-cell activation and IFN-γ production. In both AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ detection methods, these differences were statistically significant, when comparing basal versus peptide stimulation in the infected group (Fig. 2, Panels A and B and Table S6.1, Supp. data). This indicates a robust T-cell mediated response in the presence of specific peptides. In contrast, the non-infected group response to peptide stimulation showed an increase in IFN-γ production, although it did not reach statistical significance when compared to the basal condition. This suggests the presence of specific T-cells likely induced by prior vaccination against SARS-CoV-2. The application of Student's *t*-test to this group showed non-significant differences in mean values between basal and peptide-stimulated conditions (Fig. 2, Panels C and D; Table S6.2, Supp. data), further indicating that while there may be a response to the S peptides, peptides exclusively or because a lower frequency of specific T-cell precursors in peripheral blood. The observed results align with expectations considering that all administered vaccines, regardless of the manufacturer, were designed to target the Spike (S) protein or its mRNA, aiming to elicit an immune response against this specific viral protein. Additionally, the inclusion of the nucleocapsid (N) protein in this analysis is justified by recent publications which have identified it as another significant immunogenic component of the SARS-CoV-2 virus [24,25]. Increased immune responses to the N protein, which current vaccines do not target, suggests a previous infection with the virus.

For all participants in the study, significant differences were noted in the positive controls with phytohemagglutinin (PHA), compared to basal expression levels, across both detection methods, as expected considering that PHA is a mitogen to human T lymphocytes. The corresponding LFA strips for these controls are displayed in Figure S5.1, with detailed statistical analyses provided in Tables S6.1 and S6.2 of the Supp. data. Intergroup variability was observed, which could be attributed to the heterogeneity of blood leukocyte populations, as different individuals may have varying proportions of leukocyte subsets that respond differently to stimulation. Overall, the results for detecting IFN-γ production in infected individuals were comparable between AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ methods. The differences observed between infected and non-infected groups, and the sensitivity of the assays, highlight the complex interplay of factors influencing the immune response and the detection capability of different nanoparticle readout systems.

3.2. Longitudinal monitoring of SARS-CoV-2 cellular immune response

A longitudinal analysis of SARS-CoV-2-specific memory T-cell responses in infected individuals at the time of infection, and at two- and eight-months post-infection was performed. The assays measured IFN-γ production as a marker of T-cell activity in response to stimulation with SARS-CoV-2 specific peptides. At the onset of infection (0 months), significant increases in IFN-γ expression were observed in all samples upon stimulation with SARS-CoV-2 peptides compared to the basal, non-stimulated condition (which is also demonstrated in Fig. 2). This response was sustained at least up to two months post-infection, as evidenced by the results in Fig. 3, indicating the persistence of circulating memory T cells capable of responding to viral antigens. By eight months post-infection, however, the IFN-γ response to peptide stimulation diminished, as shown by the closer alignment of stimulated and basal conditions in Fig. 3. The one-way ANOVA confirmed the significant differences in the early time points (0 and 2 months), with data presented in Table S6.3 (Supp. data). For every participant across all time points, substantial differences were observed in the positive controls stimulated with PHA, when compared to their respective basal, unstimulated expression levels. These results were as expected and confirmed by both detection methods at each month interval. This consistent response serves as a validation of the assay functionality over the course of the study period. Given the reduced IFN-γ production at the eight-month mark, a Student's *t*-test was also performed to statistically confirm the differences between basal and peptide-stimulated conditions, with the results outlined in Table S6.4 (Supp. data). While T-cell responses were detected up to two months in all individuals, by eight months the responses clearly decreased, which aligns with previous studies suggesting that T-cell responses are more difficult to detect than humoral responses [24,25]. In summary, Fig. 3 demonstrates a clear initial detection and subsequent decline in SARS-CoV-2-specific T-cell responses over an eight-month period, with significant variability in response among individuals. This variability underscores the complexity of memory T-cell dynamics and the potential influence of factors such as repeated exposure to the virus.

These results are consistent and suggest that this method could be considered a novel alternative to the traditional IGRA test. The fundamentals and analytical features of current IGRA tests have been summarized and compared with the proposed solution, as detailed in Supp. Data S7 (Table S7 and Figure S7). A significant bottleneck in all *in vitro* diagnostics to speed up the results is outlined in Table S7 and relies on the incubation time required for T lymphocytes to be activated in the presence of peptides to produce cytokines *in situ.* The proposed test reduces this incubation time to 4 h by focusing on the detection of intracellular mRNA transcripts rather than released cytokines [17,26]. This approach shortens the protocol and allows the entire test to be completed in approximately 7 hours, from sample collection to result delivery. In comparison to current gold-standard techniques for tuberculosis (TB) diagnosis (Table S7, Supp. Data), such as QuantiFERON-TB Gold (QFT-GIT) and T-SPOT, which require 16–24 hours of incubation, the proposed test achieves significant time savings. In contrast, the LFA-IFN-γ assay highlights the advantages of capturing mRNA transcripts and amplifying them through end-point RT-PCR. Other detection methods such as CoVITEST with comparable time efficiency rely on detecting intracellular IFN-γ protein through cytometry [13,27]. Although CoVITEST offers high sensitivity due to the use of fluorescence readout, and the peptide-incubation in the presence of Fastimmune™ CD28/CD49d cocktail (BD Bioscience®) (a costimulatory signal to help T-cell activation allowing shorter incubation times i.e 6 h), they are often limited in application by the high cost of equipment and the specialized personnel required to operate it. An important remark related with the performance of the LFA-IFN-γ is that it could be particularly effective even under general inflammatory conditions that might elevate basal IFN-γ levels. It is designed to measure the increase in IFN-γ mRNA following antigen stimulation, ensuring specificity to the introduced antigen. This capability could be crucial in clinical settings where patients may have pre-existing inflammatory conditions or concurrent infections. By focusing on the relative increase in response to specific antigens, the test could provide accurate assessments of cellular

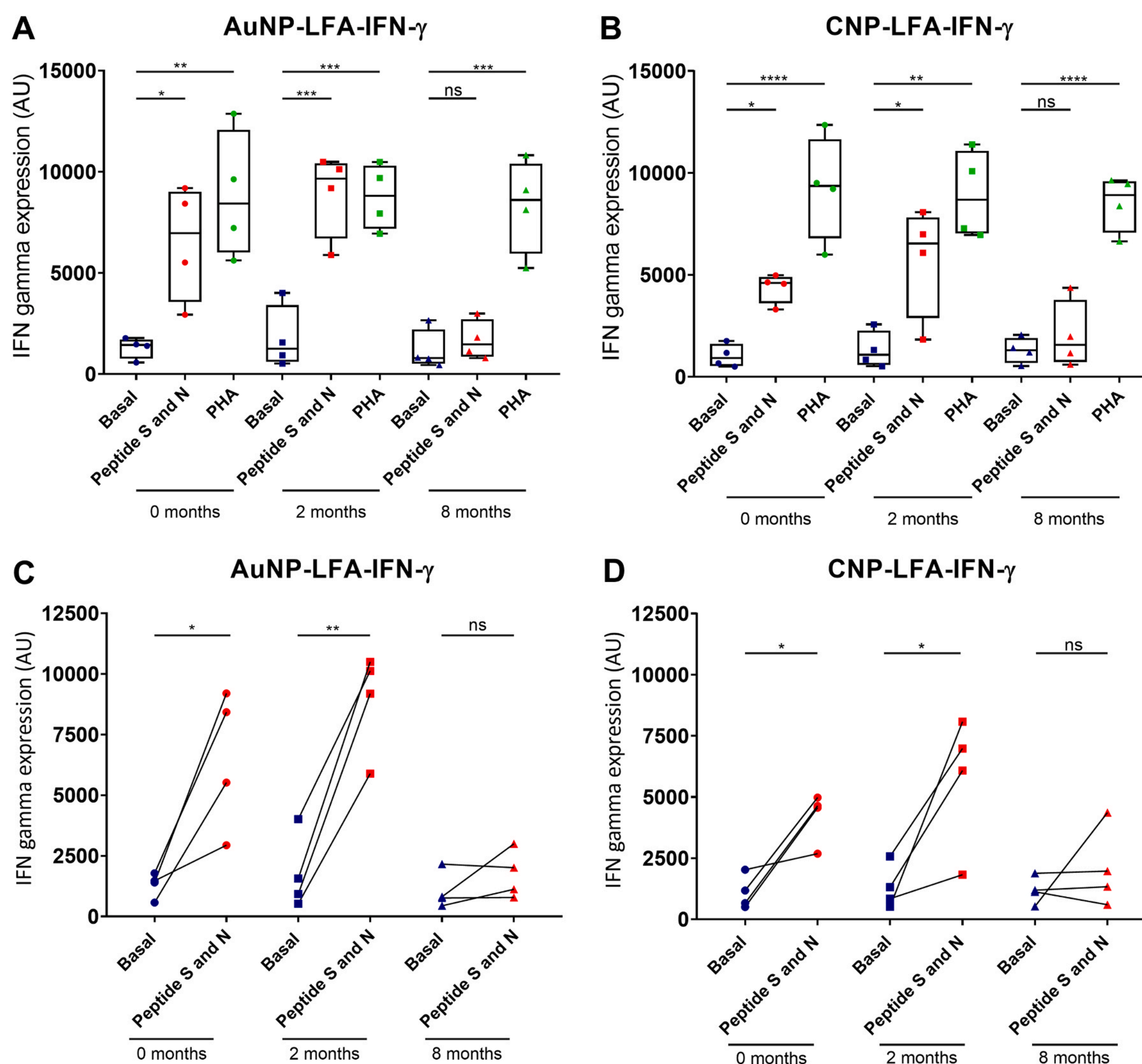


Fig. 3. Longitudinal monitoring of antigen-specific memory T-cells and IFN-γ production in infected individuals using AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ assays, illustrating the persistence of SARS-CoV-2-specific T-cell responses over time and their gradual decline. The signal obtained with the test line (displayed in Fig. S5.2, Supp. data), was processed with ImageJ software and represented here for time points at 0 months (circles), 2 months (squares), and 8 months (triangles) post-infection. Blood samples were incubated for 4 hours under three conditions: non-stimulated (blue), stimulated with S and N peptides (red), and stimulated with PHA (green). Panels A and C and panels B and D show the data from AuNP-LFA-IFN-γ and CNP-LFA-IFN-γ, respectively, in box and whiskers and before and after plots across the time points. Statistical analyses were conducted using a one-way ANOVA for panels A and B, while panels C and D were analysed using Student's t-test at the 95 % significance level. The detailed statistical results are provided in Table S6.3 and S6.4, Supp. data. Statistical significance symbols as in Fig. 2.

immunity regardless of underlying inflammation. Moreover, the LFA-IFN-γ test has potential applications beyond T cell assessments, capable of targeting a wide range of cellular and immune factors. By using specific cell surface markers, different cell types from peripheral blood can be captured and analysed. Additionally, its capability for mRNA extraction and amplification facilitates the detection of immune factors relevant to different diseases. This adaptability enhances its utility in both research and clinical diagnostics, offering a customizable platform for diverse biological markers. According to the World Health Organization, diagnostic tests must meet the criteria of ASSURED (affordable, sensitive, specific, user-friendly, rapid, and robust, equipment-free, and deliver-able to end-users) so that they can be used in low-resource environments or at the point of need [28]. Recently, the acronym was expanded to REASSURED, to include two additional criteria such as R (real-time connectivity) and E (ease of sample collection and environmental friendliness) [29]. These requirements are compatible with paper-based devices such as the lateral flow assay (LFA) combined with the use of smartphones as strip readers and CNP. Results have demonstrated that the CNP performance is comparable to the gold standard AuNP readout in the LFA, an important feature in designing affordable test. In the case of LFA-IFN-γ, although the test requires instrumentation, it does not need the sophisticated equipment often associated with qPCR-based assays. A simple, battery-operated, end-point portable thermocycler is sufficient for the test requirements. These features ensure that the test can be used effectively in settings with limited resources, thereby extending its accessibility and practical utility in various clinical environments. Moreover, the LFA readout can be linked to a smartphone integrating it with mHealth technology for results delivery and analysis.

4. Conclusions

The development of *in vitro* diagnostic tests to assess the cellular immune response has become essential due to the increasing frequency of infectious disease outbreaks and the need for patient stratification to prevent the collapse of hospital emergency services. Furthermore, such tests could provide a broader overview of the immune response for immunocompromised individuals. Therefore, this study introduces an

alternative version to the protein detection, Interferon-Gamma Release Assay based on the determination of IFN-γ transcript expression of antigen-specific T cells, LFA-IFN-γ. The methodology used in the study involved assessing cellular immune responses using peptide mixtures derived from both the spike (S) and nucleocapsid (N) proteins of the virus. This approach was crucial in identifying high response values in infected individuals while differentiating between responses due to vaccination and natural infection. Since most COVID-19 vaccines are designed to target the S protein to stimulate an immune reaction, detecting responses to this protein typically indicates vaccination. On the other hand, identifying immune responses to the N protein, which current vaccines do not target, implies a previous infection with the virus. Using both S and N antigens combined allows for a comprehensive assessment of immune responses, thereby clarifying the impact of vaccination and the presence of natural infection. Incorporating both antigens simultaneously is more efficient than using them separately, as it reduces the overall time required and simplified the test, without compromising the interpretation of the results. The LFA-IFN-γ assay, despite taking approximately 7 h in total, offers a relatively rapid alternative to existing methods like QuantiFERON-TB Gold and T-SPOT, which require 16–24 hours of incubation. By targeting mRNA transcripts instead of released cytokines, the test significantly reduces the required incubation time to 4 h and delivers results on the same day. Although PCR requires instrumentation, the test can be performed using portable, battery-operated devices, enhancing accessibility in low-resource settings. This makes it feasible for smaller labs and primary healthcare centers. The incorporation of magnetic preconcentration for CD3+ T lymphocytes reduces interference from clinical samples during stimulation by isolating the target within the containing cells. Finally, the visual readout is achieved in a paper-based LFA and CNPs, reducing costs. Due to the methodology versatility, changing the stimulating peptides allows for the evaluation of the cellular immune response to any other pathogens. This adaptability facilitates faster test development for upcoming disease outbreaks. In summary, infectious diseases are a priority in low resource settings, and the design of an alternative IGRA test amenable to a LFA is presented as a more versatile technique as it eliminates the need for expensive benchtop equipment and specialist personnel to be implemented in low resource settings.

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CRediT authorship contribution statement

Maria Isabel Pividori: Supervision, Methodology, Funding acquisition, Conceptualization. **Mercè Martí:** Supervision, Methodology, Funding acquisition, Conceptualization. Natalia Egri: Validation. **Manel Juan:** Supervision, Funding acquisition. **Europa González-Navarro:** Validation. Mireia Bernuz: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Juan Porras:** Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2024.136185.

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