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Combining spike- and nucleocapsid-based vaccines improves distal control of SARS-CoV-2

Highlights

- SARS-CoV-2 vaccines do not prevent breakthrough infection in K18-ACE2 mice
- A spike vaccine confers better protection than a nucleocapsid vaccine
- A spike vaccine confers acute protection in lung, but not in brain
- Combining spike and nucleocapsid vaccines improves distal protection in brain

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In brief

Most SARS-CoV-2 vaccines are based on the spike antigen alone, and it is unknown whether including other viral antigens improves protection. Dangi et al. show that combining a spike vaccine with a nucleocapsid vaccine improves the control of a SARS-CoV-2 infection, warranting the inclusion of nucleocapsid in next-generation SARS-CoV-2 vaccines.
Combining spike- and nucleocapsid-based vaccines improves distal control of SARS-CoV-2

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SUMMARY

SARS-CoV-2 infection causes respiratory insufficiency and neurological manifestations, including loss of smell and psychiatric disorders, and can be fatal. Most vaccines are based on the spike antigen alone, and although they have shown efficacy at preventing severe disease and death, they do not always confer sterilizing immunity. Here, we interrogate whether SARS-CoV-2 vaccines could be improved by incorporating nucleocapsid as an antigen. We show that, after 72 h of challenge, a spike-based vaccine confers acute protection in the lung, but not in the brain. However, combining a spike-based vaccine with a nucleocapsid-based vaccine confers acute protection in both the lung and brain. These findings suggest that nucleocapsid-specific immunity can improve the distal control of SARS-CoV-2, warranting the inclusion of nucleocapsid in next-generation COVID-19 vaccines.

INTRODUCTION

Most of the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) vaccines used in humans are based on the spike antigen. These vaccines have shown high efficacy against severe disease and death, but they do not always confer sterilizing immunity (Baden et al., 2021; Logunov et al., 2021; Polack et al., 2020; Sadoff et al., 2021; Voysey et al., 2021; Zhu et al., 2020). In particular, breakthrough infections can be detected in nasopharyngeal swabs of vaccinated individuals (Keehner et al., 2021; Levine-Tiefenbrun et al., 2021; Tande et al., 2021). Nasopharyngeal swabs typically contain viruses derived from the proximal site of challenge (the respiratory system), which may not reflect ongoing virus replication at distal sites of the body. It is unknown whether SARS-CoV-2 vaccines can prevent acute viral dissemination to distal sites of the body, such as the central nervous system, which is considered an immune-privileged site that is relatively “impermeable” to circulating antibodies (Forrester et al., 2018).

The central nervous system has become increasingly important in the management of coronavirus disease 2019 (COVID-19), especially because even mild or asymptomatic infections can trigger neurological manifestations, including “brain fog” and psychiatric conditions (Nalbandian et al., 2021). SARS-CoV-2 is a respiratory virus, but prior reports suggest that it may also disseminate to the brain via the olfactory mucosa (Cantuti-Castelvetri et al., 2020; Meinhardt et al., 2021; Song et al., 2021). Post-mortem analyses of COVID-19 patients have shown the presence of SARS-CoV-2 in the brain (Gasmi et al., 2021; Kumari et al., 2021; Meinhardt et al., 2021; Song et al., 2021).

Knowing whether vaccines can block viral dissemination to the central nervous system is important, because this would help elucidate whether vaccinated people who become exposed to the virus could still develop neurological complications. For ethical reasons, it is not feasible to sample the brain of vaccinated individuals to assess breakthrough infections in this distal site.

The SARS-CoV-2 spike protein is a critical antigen in coronavirus vaccines. This antigen alone is the basis for the Pfizer/BioNTek vaccine, Moderna vaccine, Johnson & Johnson’s (J&J) Janssen vaccine, AstraZeneca/Oxford vaccine, CanSino vaccine, Sputnik V vaccine, Novavax vaccine, and others. Vaccines that contain the spike antigen generate robust neutralizing antibodies that prevent the initial entry of SARS-CoV-2 into the respiratory system, but it is unclear whether other viral antigens could provide equally important immune protection. It is also unknown whether the inclusion of multiple viral antigens could provide a synergistic improvement in vaccine-elicited protection. In this study, we compared the efficacy of spike-based versus nucleocapsid-based vaccines after an intranasal SARS-CoV-2 challenge in K18-hACE2 mice, which are highly susceptible to SARS-CoV-2 infection. Surprisingly, we show that a spike-based vaccine does not provide acute protection to the central nervous system; protection against distal viral dissemination to the nervous system was observed only when a spike-based vaccine was co-administered with a nucleocapsid-based vaccine. These findings demonstrate a synergy between spike-specific and nucleocapsid-specific immune responses, and provide a framework for the rational design of next-generation coronavirus vaccines.
RESULTS

Immunogenicity of spike-based and nucleocapsid-based SARS-CoV-2 vaccines
K18-hACE2 mice constitute a robust pre-clinical model that recapitulates salient features of SARS-CoV-2 infection in humans, including respiratory insufficiency, a dysregulated inflammatory response, neurological complications, and death. Therefore, this mouse model has been useful for investigating antiviral therapies, vaccines, and COVID-19 pathogenesis (Bao et al., 2020; Oladunni et al., 2020; Rosenfeld et al., 2021; Winkler et al., 2020; Zheng et al., 2021). We vaccinated K18-hACE2 mice intra-muscularly with an adenovirus vector expressing SARS-CoV-2 spike (Ad5-S) or nucleocapsid (Ad5-N), or both (Ad5-S + Ad5-N), at a dose of 10^8 plaque-forming units (PFUs) per vector per mouse (Figure 1A).

After 3 weeks, we evaluated SARS-CoV-2-specific CD8 T cell responses in peripheral blood mononuclear cells (PBMCs) and tissues by tetramer binding assays (k^VL8 to detect spike-specific CD8 T cells; D^N219 to detect nucleocapsid-specific CD8 T cells). Ad5-S and Ad5-N vaccination elicited detectable CD8 T cell responses in PBMCs and tissues (Figures 1B–1F). We also confirmed the immunogenicity of the Ad5-S and Ad5-N vaccines by intracellular cytokine staining (ICS) assays, which showed interferon-γ (IFN-γ)-expressing CD8 T cells (Figures 2A–2C), and IFN-γ-expressing CD4 T cells (Figures 2D and 2E). Similar to prior studies with other Ad5-vectored vaccines (Larocca et al., 2016; Penaloza-MacMaster et al., 2013; Provine et al., 2016), we observed modest IFN-γ expression on virus-specific CD8 and CD4 T cells following Ad5 vaccination. The Ad5-S and Ad5-N vaccines also elicited their respective antibody responses by enzyme-linked immunosorbent assay (ELISA) (Figure 3). These results show that the spike-based and nucleocapsid-based vaccines elicit CD8 T cells, CD4 T cells, and antibody responses.

A spike-based vaccine confers proximal protection in the lung, but not distal protection in the brain
We performed intranasal challenges with 5 × 10^4 PFU of SARS-CoV-2 (isolate USA-WA1/2020), followed by euthanasia 3 days post-challenge to evaluate acute viral loads in tissues. We also challenged unvaccinated K18-hACE2 mice as controls. We compared viral loads in the lung, which represents a proximal site of challenge. Intranasal challenge of unvaccinated K18-hACE2 mice with SARS-CoV-2 normally results in high viral replication in the lung, followed by distal dissemination to the brain by day 2 post-challenge (Winkler et al., 2020). Consistent with prior studies (Oladunni et al., 2020; Rosenfeld et al., 2021; Winkler et al., 2020; Zheng et al., 2021), the unvaccinated mice showed high viral loads in the lung (Figure 4A). In contrast, mice vaccinated with the spike-based vaccine alone (but not the nucleocapsid-based vaccine alone) exhibited significant antiviral protection in the lung at day 3 post-challenge (Figure 4A).

Importantly, co-immunization of mice with the spike-based vaccine and the nucleocapsid-based vaccine did not confer any synergistic protective advantage in the lung at day 3 post-challenge, relative to spike-based vaccine alone (Figure 4A). These data show that a spike-based vaccine alone is sufficient to protect the site of initial viral entry, the respiratory system.

Effect of nucleocapsid-specific immunity in the distal control of a SARS-CoV-2 infection
At first glance, the data above suggested that nucleocapsid-specific immunity plays a dispensable role during a SARS-CoV-2 infection. Nucleocapsid-specific immunity has recently garnered attention as a main target for T cell responses (Lineburg et al., 2021; Nguyen et al., 2021; Rydzynski Moderbacher et al., 2020). Antibody responses can block the initial entry of the virus at proximal sites of challenge, but if the virus escapes the antibody response, T cell responses are critical for controlling second-round infections and subsequent viral dissemination to distal sites. SARS-CoV-2 is thought to disseminate to the nervous system after second-round infections that progress from the olfactory mucosa to the brain (Meinhardt et al., 2021). Although current spike-based SARS-CoV-2 vaccines show high efficacy in protecting the respiratory system via their induction of neutralizing antibodies, it is not clear whether they can also provide acute protection at distal sites, outside the respiratory system.

We show that mice immunized with a spike-based vaccine (or a nucleocapsid-based vaccine) did not exhibit a statistically significant improvement in antiviral protection in the brain (Figure 4B). However, combining a spike-based vaccine with a nucleocapsid-based vaccine resulted in significantly improved protection in the brain (Figure 4B). These findings show that spike-specific immunity confers acute protection at the proximal site of viral entry (the respiratory system), but acute protection at distal sites may critically depend on also having nucleocapsid-specific immunity.

DISCUSSION

Several SARS-CoV-2 vaccines have shown efficacy against SARS-CoV-2, including the Pfizer/BioNTek vaccine, Moderna vaccine, J&J Janssen vaccine, AstraZeneca/Oxford vaccine, CanSino vaccine, Sputnik V vaccine, and Novavax vaccine (Baden et al., 2021; Logunov et al., 2021; Polack et al., 2020; Sadoff et al., 2021; Voysey et al., 2021; Zhu et al., 2020). All of these vaccines are based solely on the spike protein of SARS-CoV-2, and have demonstrated high efficacy against severe COVID-19. Although these vaccines prevent severe disease and death, symptomatic infections can occur in vaccinated individuals.

Insidious neurological manifestations are common after SARS-CoV-2 infection. It is unknown whether vaccines can prevent long-term neurological manifestations of SARS-CoV-2. An unanticipated result from our studies is that a spike-based vaccine does not fully protect the central nervous system early following infection. This observation could help explain symptomatic infections that are reported in a fraction of vaccinated individuals, which can be associated with the loss of smell and other neurological manifestations.

Spike-specific antibodies are critical to prevent initial infection because they sterically block the binding of the spike protein to the host angiotensin-converting enzyme 2 (ACE2)
receptor. However, in the context of a breakthrough infection, infected cells are eliminated by cytotoxic T cells, which do not need to be spike specific to recognize infected cells. The nucleocapsid protein of SARS-CoV-2 has been suggested as an important target for T cell responses (Joag et al., 2021; Lineburg et al., 2021; Nguyen et al., 2021; Rydyznski Moderbacher et al., 2020). First, this protein contains conserved cross-reactive T cell epitopes that are present among different coronaviruses, suggesting that it could be an ideal target for universal coronavirus vaccines (Dutta et al., 2020). Second,
the nucleocapsid protein is among the most abundant structural proteins in the coronavirus life cycle (Chang et al., 2014; de Breyne et al., 2020; Weiss and Leibowitz, 2011), which may facilitate early antigen presentation and recognition by T cells.

Why does the nucleocapsid-based vaccine fail to confer acute protection at the proximal site of challenge (the lung)? It is possible that acute viral loads in the lung represent primarily viruses derived from the primary foci of infection. During the hyperacute phase, nucleocapsid-specific immune responses may not prevent breakthrough infection in the respiratory system because they target an internal protein that is not involved in viral entry. However, during subsequent second-round infections that progress from proximal sites of challenge to distal sites, nucleocapsid-specific T cells may provide a synergistic antiviral effect by killing virally infected cells, curtailing further the dissemination of the virus to distal sites. Co-immunization with a spike-based vaccine and a nucleocapsid-based vaccine accelerates the acute control of SARS-CoV-2, suggesting an advantage of including other viral antigens besides the spike protein in future COVID-19 vaccines.

A limitation of our study is that we only measured viral loads at a very early time point. We did not compare viral loads at later time points, because unvaccinated animals succumb (or must be euthanized) after day 5 post-challenge (Oladunni et al., 2020), and because it is already known that spike-based vaccines elicit immune responses that ultimately clear SARS-CoV-2 within 1 week of challenge (Feng et al., 2020; Wu et al., 2020). Our study was focused on evaluating hyperacute viral control to assess breakthrough infection. Future studies will assess the contribution of nucleocapsid-specific antibodies versus T cells, and whether vaccines that encode other viral antigens (e.g., envelope, membrane) can improve further distal protection after a SARS-CoV-2 challenge. Although the K18-hACE2 model is considered useful to evaluate vaccines and antivirals, it is possible that the extensive viral dissemination in the brain may be a peculiarity of this mouse model. Nevertheless, it is still reasonable to conclude that nucleocapsid-specific immunity improves the acute distal control of SARS-CoV-2. In conclusion, we show a substantial benefit of including both spike and nucleocapsid antigens in COVID-19 vaccines. These
results warrant the incorporation of nucleocapsid in future coronavirus vaccines.

**ACKNOWLEDGMENTS**

We thank Drs. Susan Weiss, Stanley Perlman, Thomas Gallagher, David Masopust, and Vaiva Vezys for discussions and reagents. This work was possible with startup funds to J.M.R., a grant from the Emerging and Re-Emerging Pathogens Program (EREPP) at Northwestern University to P.P.M., and a grant from the National Institute on Drug Abuse (NIDA, DP2DA051912) to P.P.M.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| αCD8, clone 53-6.7 in PerCP-Cy5.5 | BD PharMingen | 551162 |
| αCD4, clone IM7 in Pacific Blue | Biolegend | 103020 |
| αIFNγ, clone XMG1.2 in APC | BD PharMingen | 554413 |
| αCD107a, clone 1D4B in FITC | BD PharMingen | 553793 |
| αCD4, clone RM4-5 FITC | eBioscience | 11-0042-82 |
| **Bacterial and virus strains** | | |
| Ad5-SARS-CoV-2 N | A gift from Dr. David Masopust at University of Minnesota | N/A |
| Ad5-SARS-CoV-2 S | University of Iowa Vector Core, custom made | N/A |
| SARS-Related Coronavirus 2, Isolate USA-WA1/2020, | BEI resources | NR-52281 |
| **Chemicals, peptides, and recombinant proteins** | | |
| SARS-CoV-2 S protein overlapping peptide pools (181 peptides) | BEI resources | NR-52402 |
| SARS-CoV-2 N protein overlapping peptide pools (59 peptides) | BEI resources | NR-52404 |
| VL8 (VNFNFNGL) | GenScript | N/A |
| N219 (LALLLDRL) | GenScript | N/A |
| SARS CoV-2 S protein for ELISA | Made by the Northwestern University recombinant protein production core using a SARS CoV-2 S protein gene provided by BEI (NR-52394, see above) | N/A |
| SARS CoV-2 N protein for ELISA | BEI resources | NR-53797 |
| **Critical commercial assays** | | |
| Quick RNA 96 kit (RNA extraction) | Zymo Research | R1052 |
| Taqman RNA-to-CT step kit | ThermoFisher | 4392653 |
| **Experimental models: Cell lines** | | |
| HEK293 | ATCC | CRL-1573 |
| Vero-E6 | ATCC | CRL-1586 |
| **Experimental models: Organisms/strains** | | |
| K18-hACE2 mice | Jackson Laboratories | 034060 |
| **Recombinant DNA** | | |
| Plasmid containing SARS CoV-2 spike (S) gene | BEI resources | NR-52394 |
| Plasmid containing SARS CoV-2 nucleocapsid (N) gene | BEI resources | NR-53507 |
| **Software and algorithms** | | |
| GraphPad Prism software version 9.1.0 | [https://www.graphpad.com/scientific-software/prism/](https://www.graphpad.com/scientific-software/prism/) | N/A |
| FlowJo version 10 | [https://www.flowjo.com](https://www.flowjo.com) | N/A |
| **Other** | | |
| KβVL8 tetramer (APC) | Provided by the NIH tetramer core at Emory University | N/A |
| KβN219 tetramer (PE) | Provided by the NIH tetramer core at Emory University | N/A |
| Forward primer, 5’ GACCCCAAAATCAGCCGAAAT 3’ | Integrated DNA Technologies | 10006713 |
| Reverse primer, 5’ TCTGTTTACTGCAGTTGAATCTG 3’ | Integrated DNA Technologies | 10006713 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contacts
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Pablo Penaloza-MacMaster (ppm@northwestern.edu) and/or Justin Richner (richner@uic.edu).

Materials availability
For Ad5-SARS CoV-2 spike viral vector access, contact the University of Iowa vector core at vector@uiowa.edu. For Ad5-SARS CoV-2 nucleocapsid viral vector access, contact David Masopust at masopust@umn.edu. For VNFNFNLG and LALLLLDRL peptides, contact GenScript at sales.US@genscript.com.

Data and code availability
This article does not report original code. The article includes all the datasets and analyses generated for this study. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Animals and ethics statement
Mice were purchased from Jackson laboratories and were housed at Northwestern University or University of Illinois at Chicago (UIC) animal facility. All procedures were performed with the approval of the center for comparative medicine at Northwestern University and the UIC IACUC. Adult mice, approximately half females and half males were used for the immunogenicity experiments included in this study. For the challenge studies, female mice were used.

METHOD DETAILS

Cell lines
Adenoviral vectors were propagated using HEK293 cells purchased from ATCC (cat # CRL-1573). Vero E6 cells were used to propagate SARS CoV-2 isolate USA-WA1/2020 (BEI resources, NR-52281). Cells were not authenticated as they were purchased from a reputable vendor and certificate of analysis was obtained.

Mice and vaccinations
6-8-week-old K18-hACE2 mice were used. These mice express the human ACE2 protein behind the keratin 18 promoter, directing expression in epithelial cells. Mice were purchased from Jackson laboratories (Stock No: 034860). Mice were immunized intramuscularly (50 μL per quadriceps) with an Ad5 vector expressing SARS-CoV-2 spike protein (Ad5-S), or nucleocapsid protein (Ad5-N), or both vectors combined; diluted in sterile PBS, at 10^9 PFU per mouse. Ad5-N was a kind gift of the Masopust/Vezys laboratory (Joag et al., 2021). These are non-replicating Ad5 vectors (E1/E3 deleted). The vectors contain a CMV (Cytomegalovirus) promoter driving the expression of the respective proteins. The Ad5 vectors were propagated on trans-complementing HEK293 cells (ATCC), purified by cesium chloride density gradient centrifugation, titrated, and then frozen at −80 °C.

SARS-CoV-2 virus and infections
SARS-Related Coronavirus 2, Isolate USA-WA1/2020, NR-52281 was deposited by the Centers for Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH. Virus was propagated and tittered on Vero-E6 cells (ATCC). In brief, Vero cells were passaged in DMEM with 10% Fetal bovine serum (FBS) and Glutamax. Cells less than 20 passages were used for all studies. Virus
stocks were expanded in Vero-E6 cells following a low MOI (0.01) inoculation and harvested after 4 days. Viral titers were determined by plaque assay on Vero-E6 cells. Viral stocks were used after a single expansion (passage = 1) to prevent genetic drift.

K18-hACE2 mice were anesthetized with isoflurane and challenged with $5 \times 10^4$ PFU of virus intranasally. Mouse infectious were performed at the University of Illinois at Chicago (UIC) following BL3 guidelines with approval by the UIC Institutional Animal Care and Use Committee (IACUC).

**SARS-CoV-2 quantification in tissues**

Tissues were isolated from infected mice and homogenized in sterile PBS. RNA was isolated with the Zymo 96-well RNA isolation kit (Catalog #: R1052) following the manufacturer’s protocol. SARS-CoV-2 viral burden was measured by RT-qPCR using Taqman primer and probe sets from IDT with the following sequences: Forward 5' GAC CCC AAA ATC AGC GAA AT 3', Reverse 5' TCT GTT TAC TGC CAG TTG AAT CTG 3', Probe 5' ACC CCG CAT TAC GTT TGG ACC 3'. A SARS-CoV-2 copy number control was obtained from BEI (NR-52358) and used to quantify SARS-CoV-2 genomes.

**Reagents, flow cytometry and equipment**

Single cell suspensions were obtained from PBMCs or tissues. Dead cells were gated out using Live/Dead fixable dead cell stain (Invitrogen). SARS-CoV-2 nucleocapsid or spike peptides were used for intracellular cytokine staining (ICS) to detect nucleocapsid- and spike-specific CD8 T cells. The peptide stimulation was performed in the presence of GolgiPlug and GolgiStop (BD Biosciences) for 5 hr at 37°C in a 5% CO₂ incubator. MHC class I monomers (KβVL8, VNFNFNGL and Kβ219, LALLLDRL) were used for detecting virus-specific CD8 T cells, and were obtained from the NIH tetramer facility located at Emory University. MHC monomers were tetramerized in-house. The VNFNFNGL epitope is located in position 539-546 of the SARS-CoV-2 spike protein. Cells were stained with fluorescently-labeled antibodies against CD8α (53-6.7 on PerCP-Cy5.5), CD44 (IM7 on Pacific Blue), KβVL8 (APC), KβN219 (PE), CD107a (1D4B on FITC), and IFNγ (XMG1.2 on APC). Fluorescently-labeled antibodies were purchased from BD PharMingen, except for anti-CD44 (which was from Biolegend). Flow cytometry samples were acquired with a Becton Dickinson Canto II or an LSRII and analyzed using FlowJo v10 (Treestar).

**SARS-CoV-2 spike and nucleocapsid specific ELISA**

Binding antibody titers were quantified using ELISA as described previously (Dangi et al., 2020; Palacio et al., 2020), using spike protein and nucleocapsid protein as coating antigens. 96-well flat bottom plates MaxiSorp (Thermo Scientific) were coated with 0.1 μg/well of SARS-CoV-2 spike protein or nucleocapsid protein, for 48 hr at 4°C. Plates were washed with PBS + 0.05% Tween-20. Blocking was performed for 4 hr at room temperature with 200 μL of PBS + 0.05% Tween-20 + bovine serum albumin. 6 μL of sera were added to 144 μL of blocking solution in first column of plate, 1:3 serial dilutions were performed until row 12 for each sample and plates were incubated for 60 minutes at room temperature. Plates were washed three times followed by addition of goat anti-mouse horseradish peroxidase-conjugated IgG (Southern Biotech) diluted in blocking solution (1:5000), at 100 μL/well and incubated for 60 minutes at room temperature. Plates were washed three times and 100 μL/well of Sure Blue substrate (Sera Care) was added for approximately 8 minutes. Reaction was stopped using 100 μL/well of KPL TMB stop solution (Sera Care). Absorbance was measured at 450 nm using a Spectramax Plus 384 (Molecular Devices). SARS-CoV-2 spike protein was made at the Northwestern Recombinant Protein Production Core by Dr. Sergii Pshenychnyi using plasmid that was produced under HHSN272201400008C and obtained through BEI Resources, NIAID, NIH: Vector pCAGGS Containing the SARS-Related Coronavirus 2, Wuhan-Hu-1 Spike Glycoprotein Gene (soluble, stabilized), NR-52394. SARS-CoV-2 nucleocapsid protein was obtained through BEI Resources, NIAID, NIH: Nucleocapsid Protein from SARS-Related Coronavirus 2, Wuhan-Hu-1 with C-Terminal Histidine Tag, Recombinant from Baculovirus, NR-53797.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses are indicated on the figure legend. Dashed lines in data figures represent limit of detection. Statistical significance was established at $p \leq 0.05$ and was generally assessed by two tailed unpaired Student’s t tests, unless indicated otherwise in figure legends. Data were analyzed using Prism (Graphpad).