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Lung-resident T cells elicited by SARS-CoV-2 do not mediate protection against secondary infection

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Abstract

Immunity to pulmonary infection typically requires elicitation of lung-resident T cells that subsequently confer protection against secondary infection. The presence of tissue resident T cells in SARS-CoV-2 convalescent patients is unknown. Using a sublethal mouse model of COVID-19, we determined if SARS-CoV-2 infection potentiated antigen-specific pulmonary resident $CD4^+$ and $CD8^+$ T cells responses and if these cells mediated protection against secondary infection. S protein specific T cells were present in resident and circulating populations. However, M and N protein specific T cells were only detected in the resident T cell pool. Using an adoptive transfer strategy, we found that T cells from SARS-CoV-2 immune animals did not protect naïve mice. These data indicate resident T cells are elicited by SARS-CoV-2 infection but are not sufficient for protective immunity.

INTRODUCTION

Establishing correlates of protection is a critical step in vaccine development. The focus of current vaccine strategies to prevent SARS-CoV-2 infection, the causative agent of COVID-19, has centered on driving high neutralizing antibody titers to block viral entry into host cells [1]. While the protective nature of antibodies in COVID-19 is well established, the role of T cells in immunity, particularly those resident in the lung tissue, remains a lingering question [2]. Antigen-specific $CD4^+$ and $CD8^+$ T cells have been detected in the peripheral blood of COVID-19 convalescent patients [3, 4]. However, it is unclear whether SARS-CoV-2 infection elicits a pulmonary resident T cell response and if T cells alone are sufficient to prevent lethal disease.

Understanding the contributions of specific immune subsets to protection requires the use of animal models of disease where antibodies or cells can be added or eliminated prior to infection. Convalescent human plasma protects mice expressing the human angiotensin converting enzyme 2 (hACE2) from lethal SARS-CoV-2 infection while purified convalescent IgG from rhesus macaques decreased viral loads using both prophylactic

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and therapeutic treatment strategies [5, 6]. These passive transfer studies confirmed the protective nature of antibodies in COVID-19. Recently, the role of $CD8^+$ T cells in convalescent non-human primates has also been addressed. Convalescent rhesus macaques depleted of their $CD8^+$ T cells had partially impaired control of SARS-CoV-2 in the upper respiratory track indicating $CD8^+$ T cells play a role in restricting viral replication [5]. Similarly, depletion of $CD4^+$ and $CD8^+$ T cells during primary SARS-CoV-2 infection in rhesus macaques delayed viral clearance, but the animals ultimately cleared the infection and the response to secondary challenge was not impacted [7]. These data suggest T cells play a role in viral clearance and may be important as antibody responses wane over time, but notably, did not address whether T cells alone are sufficient for protective immunity.

When evaluating the role T cells are playing in the immune response, it is essential to consider the anatomical location of the response. Pulmonary resident T cells are retained in the airway and/or lung parenchyma after a variety of viral and bacterial infections and are positioned to swiftly respond to secondary respiratory exposure to pathogens [8–13]. Underscoring the critical role tissue resident T cells play in defense against infection, it has been demonstrated by several groups that these cells alone are sufficient for survival of multiple acute viral and bacterial infections [11–13]. In contrast to the tissue resident T cells which rapidly respond to localized infection, circulating T cells replenish the resident pool to either maintain immunity or work in concert with resident T cells to control disseminated infection [8, 10]. Together, these examples highlight the importance of identifying the location of T cells elicited by respiratory infection and their contribution to protective immunity as expansion of appropriate pools of T cells will dictate both vaccine design and delivery. Herein, we established a sublethal SARS-CoV-2 primary infection model in hACE2 mice to determine if natural infection promoted establishment of SARS-CoV-2 specific resident T cells and, if present, what contribution these cells made to survival of secondary lethal infection.

MATERIAL AND METHODS

SARS-CoV-2

SARS-CoV-2 USA/WA1/2020 was obtained from BEI Resources. Viral stocks were generated in Vero cells and frozen at −80°C until use.

Mice and inoculations

Specific-pathogen free, 6-10 week old, male and female B6.Cg-Tg(K18-ACE2)2Prlmn/J (K18-hACE2; stock #034860) mice were purchased from The Jackson Laboratory and/or bred in-house. For SARS-CoV-2 infections, mice were intranasally inoculated with 10^1-10^4 PFU in 25 μl PBS to a single nare after anesthetizing with ketamine/xylazine. Prior to euthanizing animals for tissue harvest, mice were anesthetized with ketamine/xylazine and then retro-orbitally injected with 2.5 μg CD45.2 FITC (Biolegend) in 100 μl PBS to label circulating cells. All animal studies were approved by and conducted in accordance with Rocky Mountain Laboratories' Animal Care and Use Committee under animal study protocol 2020-075-E.

Tissue harvest and processing

Lungs were aseptically removed after intravascular staining of circulating cells and subsequent euthanasia. Single cell suspensions were generated as previously described [8]. Briefly, lungs were minced and then digested with Liberase TM (Sigma-Aldrich). Red blood cells were lysed with ACK lysis buffer (Life Technologies) and the total number of viable cells was determined using trypan blue exclusion using a TC20 automated cell counter (Bio-Rad).

Flow cytometry analysis

Single cell suspensions were stained with Zombie Near-IR (Biolegend) to distinguish live and dead cells. Cells were stained with the following antibodies (Biolegend or BD Biosciences): CD3 BUV395, CD4 AF700, CD8 BV605, CD44 PE-Cy7, CD62L Pacific Blue, CD69 PE, CD103 APC, IFN-γ PE, TNF-α APC, and/or IL-2 Pacific Blue. Prior to intracellular cytokine staining, 1.5E6 lung cells were stimulated for 5 hours with Influenza A NP or SCV2 M, N, or S+ Peptivator pools (Miltenyi Biotec) or for 4 hours with PMA/ionomycin (Biolegend). During the last 4 hours of culture, brefeldin A (Biolegend) was added to inhibit cytokine secretion. Data acquisition was performed on a Symphony Flow Cytometer (BD Biosciences) and data analyzed using FlowJo 10 (BD Biosciences). The gating scheme utilized for analysis, including assessment of cytokine production among cells stimulated with peptide pools is shown in Supplemental Figs. 1 and 2.

Adoptive transfer of T cells

Total pulmonary T cells were isolated from the naïve hACE2 animals or total pulmonary T_{eff} from SARS-CoV-2 immune hACE2 mice via cell sorting using the MACSQuant Tyto (Miltenyi Biotec). Where indicated, 3.5E5 cells were intratracheally transferred into naïve hACE2 mice in 50 μl of sterile saline or 1.4E6 cells were intravenously transferred into naïve hACE2 mice in 100 μl of sterile saline. Twenty-four hours later, mice were intranasally challenged with either 10^3 or 10^2 PFU of SARS-CoV-2 and survival determined.

Statistical analysis

Statistically significant differences between two groups were determined using an unpaired two-tailed *t* test. For T cell cytokine production, a paired two-tailed t test was utilized to compare the mock and peptide stimulated samples. In all cases, statistical significance was set at $p < 0.05$. Significant differences in survival between groups was determined using a log-rank (Mantel-Cox) test.

RESULTS AND DISCUSSION

Dose-dependent survival of K18-hACE2 mice after SARS-CoV-2 infection

We first determined the LD_{50} of SARS-CoV-2 in hACE2 mice to identify doses of infection that resulted in either sub-lethal or lethal intranasal infection. Animals inoculated with 10^3 or 10^4 PFU required euthanasia by day 6 post-challenge (Fig. 1A). Approximately 50% and 20% of animals infected with 10^2 or 10^1 PFU, respectively, succumbed to infection (Fig. 1A). Thus, the calculated LD_{50} was 92 PFU. The susceptibility of hACE2 mice to

higher doses of SARS-CoV-2 in our hands is consistent with a previous report [14]. We next determined if sublethal SARS-CoV-2 infection induced protective immunity against lethal secondary challenge. Twenty-eight days after primary infection, animals were re-infected with 10^3 PFU. As expected, all naïve controls succumbed by day 7 post-challenge (Fig. 1B). In contrast, 100% of animals previously infected with 10^2 PFU survived secondary lethal challenge (Fig. 1B). Given the presence of complete protective immunity at a primary infection dose of 10^2, we utilized this as an immunizing dose for all subsequent studies.

Infection with SARS-CoV-2 elicits tissue resident T cell responses

Pulmonary infections often lead to the recruitment and retention of T cells into the lung establishing a pool of tissue resident effector T cells. These cells are critical for defense against a variety of pathogens whereas T cells circulating through the lung via the vasculature are dispensable [11–13, 15]. However, infection in the pulmonary compartment is no guarantee that pools of tissue resident effector T cells will develop. For example, we have observed that animals which survived intranasal *Klebsiella. pneumoniae* infection and are 100% protected against lethal secondary challenge do not have detectable tissue resident T cells in their lungs (unpublished observation). It is unknown if SARS-CoV-2 infection triggers expansion of resident T cells. Moreover, if these cells are present, it is also unknown if antigen specificity among resident effector T cells is similar to those in circulation. Therefore, we next determined if sublethal SARS-CoV-2 infection resulted in generation of pulmonary resident effector T cells followed by identification of their antigen specificity. In contrast to naïve controls, both resident $CD4^+$ and $CD8^+$ effector T cells ($CD44^{hi}$; T_{eff}) were detected in SARS-CoV-2 lungs 28 days after infection (Fig. 2A–D). The resident T cells from SARS-CoV-2 infected mice expressed markers associated with tissue-resident memory including CD69 and CD103 (Fig. 2E, F). These immune animals also had significantly greater numbers of circulating $CD4^+$ and $CD8^+$ T_{eff} (Fig. 2G, H). Interestingly, the magnitude of the response to SARS-CoV-2 (10-25% resident of $CD4^+$ or $CD8^+$ T cells) was lower than that observed for other pulmonary viral and bacterial infections. For example, 50-80% of $CD4^+$ and $CD8^+$ T cells after Influenza A and >45% of $CD4^+$ following *B. pertussis* infection are tissue resident T cells [11, 12]. Notably, Influenza A and *B. pertussis* persist in the lung for much longer than SARS-CoV-2. Thus, one possibility for the paucity of resident T cells present after SARS-CoV-2 infection could be a consequence of its rapid clearance from the host which negates the requirement for a strong T cell response to ultimately control and clear the infection.

Resident and circulating T cells are specific for M, N, and S antigens

The presence of increased numbers of resident and circulating $CD4^+$ and $CD8^+$ T_{eff} in SARS-CoV-2 immune animals led us to ask which antigen(s) these cells were responding to. Both $CD4^+$ and $CD8^+$ T cells isolated from the peripheral blood of COVID-19 patients produce Th1 cytokines IFN-γ and TNF-α in response to antigen stimulation [3, 4]. Therefore, we compared the ability of pulmonary resident and circulating T cells from SARS-CoV-2 immune mice to produce these cytokines in response to non-specific and antigen-specific stimuli. All T cell subsets readily produced both IFN-γ and TNF-α in response to non-specific stimulation with PMA and ionomycin demonstrating SARS-CoV-2 infection in mice also drives a strong Th1 response (Supplemental Fig. 3). To

identify antigen-specific responses, immune lung cells were stimulated with overlapping peptide pools from either Influenza A NP (negative control) or SARS-CoV-2 M, N, or S. The response to each peptide was directly compared within each immune mouse to the corresponding mock stimulated sample. Immune circulating $CD4^+$ T cells produced IFN-γ in response to S (Fig. 3A). Within the resident $CD4^+$ pool, responses specific for M, N, and S were detected (Fig. 3B, C). Resident $CD8^+$ T cells responded to S and there was a trend towards a response to N, but the difference did not reach statistical significance (Fig. 3E). Notably, only $CD4^+$ T cells within the resident pool produced cytokine in response to M and N (Fig. 3B, C). Our data extend the studies using human peripheral blood by examining antigen specificity within pulmonary resident T cells and revealed circulating T cells shared specificity for some, but not all, antigens. Thus, while admittedly challenging, the ability to examine tissue resident T cell responses in human lungs at autopsy or airway cells obtained by bronchoalveolar lavage among convalescent patients provides an important opportunity to understand the pulmonary immune response not only to SARS-CoV-2, but other infections as well [16]. Together these data suggest anatomical antigen-specificity for T cell responses to SARS-CoV-2 and underscores the utility of including multiple antigens in future vaccine iterations. Further, the use of several antigens could lessen the chance that viral mutations in a single antigen would render a vaccine ineffective.

Adoptive transfer of pulmonary T cells does not protect against SARS-CoV-2 challenge

Since we observed the presence of tissue resident effector T cells in lungs of SARS-CoV-2 mice we next addressed whether these cells could independently protect the naïve host against lethal SARS-CoV-2 infection. Therefore, we adoptively transferred immune T cells into a naïve host prior to SARS-CoV-2 infection. Due to the limited number of T_{eff} in naïve animals, mice that received total pulmonary T cells purified from naïve lungs served as negative controls. We purified total T_{eff} from SARS-CoV-2 immune animals because we reasoned SARS-CoV-2-specific T cells would be enriched within the T_{eff} pool giving us optimal opportunity to assess whether immune T cells were sufficient for protective immunity. Initially, we challenged mice with 10^3 PFU following adoptive transfer of either naïve T cells or immune T_{eff} into naïve hosts. We observed equivalent lethality in both groups indicating T cells were not sufficient for protection against this dose of SARS-CoV-2 (data not shown). It was possible that T cells may make a minor contribution to control of secondary SARS-CoV-2 infection that was overwhelmed by a high challenge dose. Therefore, we examined the ability of adoptively transferred T_{eff} could mediate protection against an approximate LD_{50} challenge. One day after transfer of naïve T cells or immune T_{eff} cells mice were intranasally challenged with 10^2 PFU SARS-CoV-2. Following infection, 20% of animals receiving naïve donor T cells succumbed to SARS-CoV-2 infection (Fig. 4A). In contrast to control animals, there was increased mortality in the immune T_{eff} group (40%) compared to those receiving naïve T cells, however this difference was not statistically significant (Fig. 4A). Due to the small volume necessary for intratracheal adoptive transfer, we limited the number of cells given to prevent aggregates that could block airways. Ultimately, this led to fewer cells transferred than are present in an immune lung (Fig. 2). Thus, we transferred almost five times as many immune T_{eff} intravenously to reflect the number of resident and circulating T_{eff} present in the immune lung and then infected with 10^2 PFU of SARS-CoV-2 one day later. As observed with

intratracheal transfer, immune T_{eff} were unable to significantly improve survival compared to naïve animals (Fig. 4B). Together, these data indicate immune effector T cells were not sufficient for protective immunity and at lower challenge doses, may be pathogenic when cells are delivered directly into the airspace.

Our finding that T cells did not independently confer protective immunity against to SARS-CoV-2 is in contrast to previous reports examining immune requirements following infection with SARS. Specifically, airway-resident $CD4^+$ T cells were critical for vaccine-mediated immunity to mouse adapted SARS in Balb/c mice [13]. However, depletion of $CD4^+$ T cells in that study did not result in uniform lethality. This suggested that while T cells contribute to protection other immune cell subset(s) also actively participate in protective immunity [13]. The disconnect between our data and this earlier SARS work could be a consequence of the strategy used to generate immune T cells. In the study cited above immune animals were generated by using a vaccine directed against SARS. It is possible that unlike a natural infection the vaccine promoted an immune response in which T cells gained the capability to more actively participate in defense against infection. Another possibility is that there were inherent difference in the development of immunity based on the strain of mouse used in the study (hACE2 B6 versus Balb/c) and/or differences in the underlying protective mechanisms required for SARS versus SARS-CoV-2. Another possibility for the inability of adoptively transferred immune T_{eff} to confer protective immunity in our model of SARS-CoV-2 infection could be their restriction to the lung. While we reasoned adoptively transferring total T_{eff} from immune animals into the airways of naïve hACE2 mice would allow these cells optimal interaction with infected epithelial cells immediately after SARS-CoV-2 infection, SARS-CoV-2 can infect organs outside of the lung [14]. The consequences of extra-pulmonary SARS-CoV-2 infection to disease lethality is still under debate but limiting the T cell response to the airway could be detrimental for controlling viral replication in other locations. In addition to the possibility that extra- pulmonary infection contributed to the lack of function for pulmonary immune T cells in protection against SARS-CoV-2, the function of adoptively transferred T cells could also have been negatively impacted by alveolar macrophages (AMs). AMs negatively regulate dendritic cell function and in the context of SARS infection, suppress activation and migration of dendritic cells which are responsible for initiating anti-SARS T cell responses [17–19]. Finally, transferred T cells may not receive optimal cytokine signaling due to the acute nature of SARS-CoV-2 infection in mice coupled with dampened inflammatory cytokine responses that are necessary to potentiate existing T cell responses [14].

Although T cells were not solely responsible for the protective immune response, they likely remain an important component of the response to SARS-CoV-2. Indeed, $CD8^+$ T cell depletion in convalescent rhesus macaques revealed this cellular subset is required for optimal viral control [5]. These data raise the possibility that resident T cells could coordinate the overall immune response in the tissue as demonstrated in other infectious models [20–22]. For example, a newly identified population of lung resident $CD4^+$ T cells termed resident helpers given their shared characteristics of T follicular helper cells and resident memory T cells, coordinates the response of B cells and $CD8^+$ T cells in Influenza A [22]. Likewise, skin resident $CD4^+$ T cells coordinate the recruitment of circulating T cells to control *Leishmania* infection [20]. Resident T cells may act similarly as cellular

conductors within the lung to efficiently coordinate the diverse response that eliminates SARS-CoV-2. While it remains to be seen whether T cells are long-lived after SARS-CoV-2, anti-SARS T cells were found in convalescent humans after antibody responses were no longer detectable [15, 23–25]. Thus, eliciting long-lived, antigen-diverse T cell responses by future vaccine candidates may be a critical component for provoking durable immunity against COVID-19.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

1. SARS-CoV-2 infection elicits pulmonary resident $CD4^+$ and $CD8^+$ T cells
2. Pulmonary T cells are not sufficient for protection against secondary SARS-CoV-2

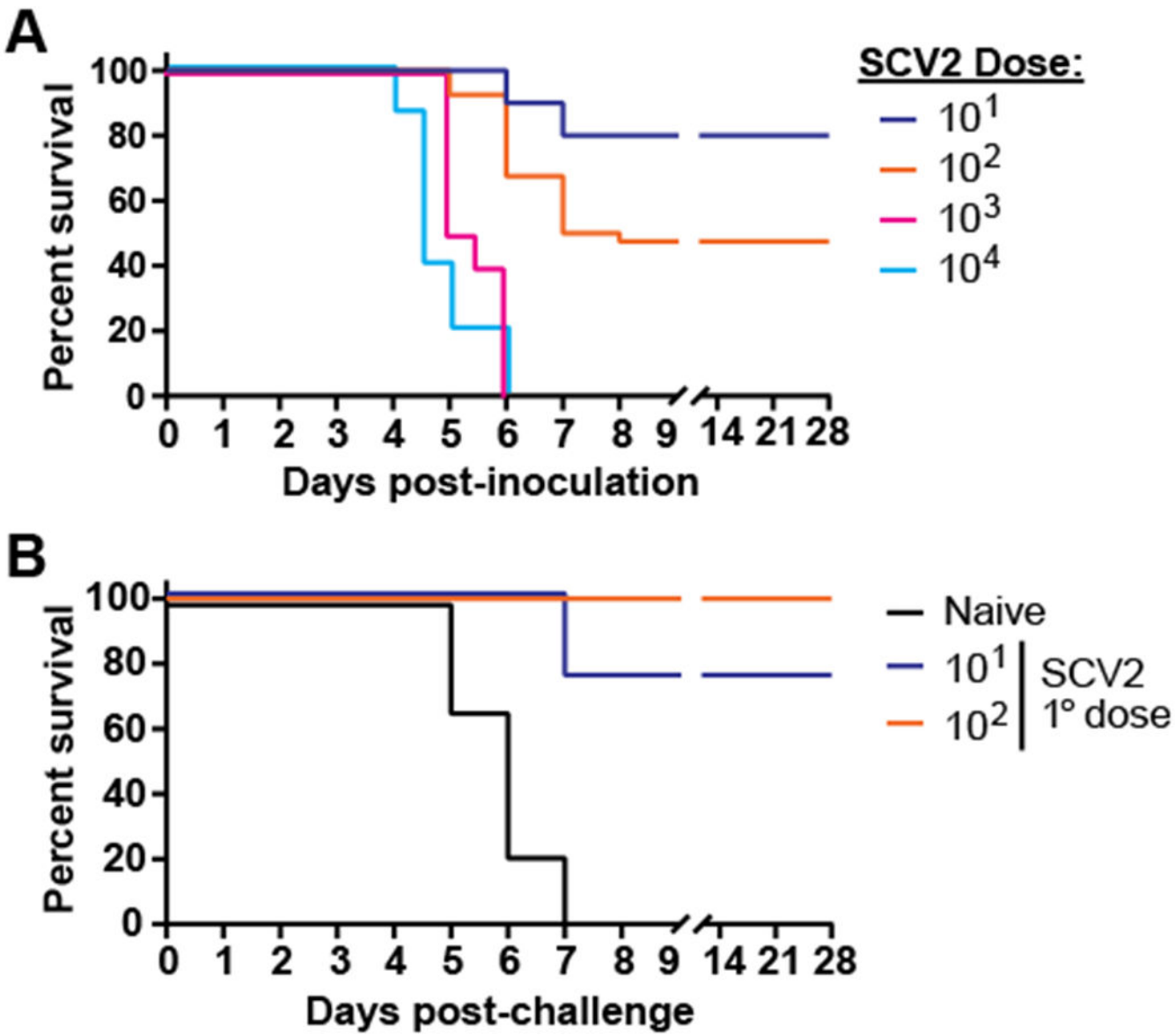

Figure 1. Dose-dependent survival of hACE2 mice after SARS-CoV-2 infection.

A) hACE2 mice were intranasally infected with increasing doses of SARS-CoV-2 and were monitored for signs of illness. n=10-40 mice/group with data combined from 6 independent experiments. B) Naïve animals or mice that survived 10^1 or 10^2 PFU primary infection with of SARS-CoV-2 were intranasally challenged with 10^3 PFU 28 days after the initial infection and were monitored for signs of illness. n=4-9 mice/group with data combined from 2 independent experiments.

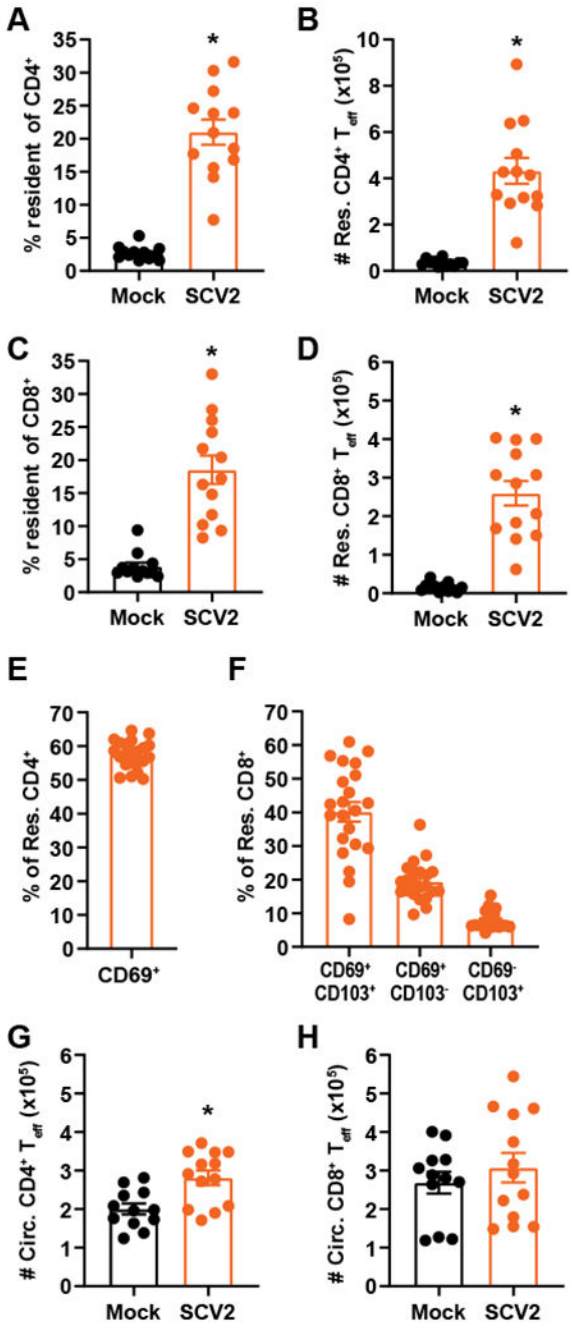

Figure 2. Infection with SARS-CoV-2 elicits tissue resident T cell responses.

hACE2 mice were intranasally infected with 10^2 PFU of SARS-CoV-2. Twenty-eight days later, the A) percent resident of $CD4^+$, B) number of resident $CD4^+$ T_{eff}, C) percent resident of $CD8^+$, D) number of resident $CD8^+$ T_{eff}, E) percent $CD69^+$ of resident $CD4^+$ from SCV2 infected mice, F) percent expressing CD69 and/or CD103 of resident $CD8^+$ from SCV2 infected mice, G) number of circulating $CD4^+$ T_{eff}, and H) number of circulating $CD8^+$ T_{eff} was determined using flow cytometry. Uninfected mice served as negative controls. n=12-13 mice/group and are combined from 3 independent experiments for A-D, G, H; n=22 mice

combined from 2 independent experiments for E, F. Each point represents an individual mouse; error bars indicate SEM. Statistical significance was determined using an unpaired t-test; * indicates $p < 0.05$.

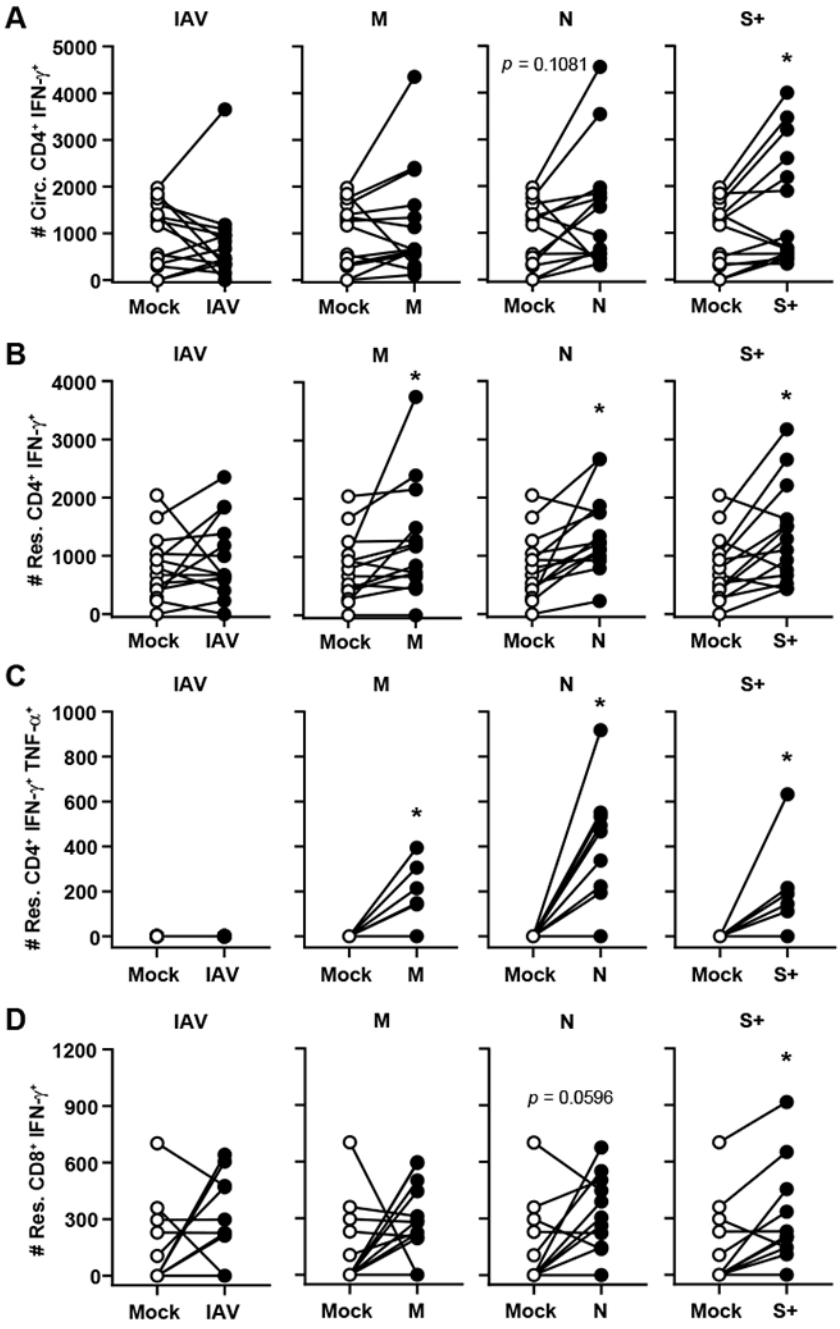

Figure 3. Resident and circulating T cells are specific for M, N, and S antigens.

hACE2 mice were intranasally infected with 10^2 PFU of SARS-CoV-2. Twenty-eight days later, lung cells were mock stimulated, stimulated with PMA/Ionomycin, or IAV NP, SCV2 M, SCV2 N, or SCV2 S+ peptide pools. The production of A) IFN-γ by circulating $CD4^+$ T cells, B) IFN-γ by resident $CD4^+$ T cells, C) IFN-γ and TNF-α by resident $CD4^+$ T cells, and D) IFN-γ by resident $CD8^+$ T cells was determined by flow cytometry. n=14 mice/group and are combined from 3 independent experiments. Each point represents an individual mouse; error bars indicate SEM. Statistical significance was determined using a

paired two-tailed t test to compare the mock and peptide stimulated samples; * indicates $p < 0.05$.

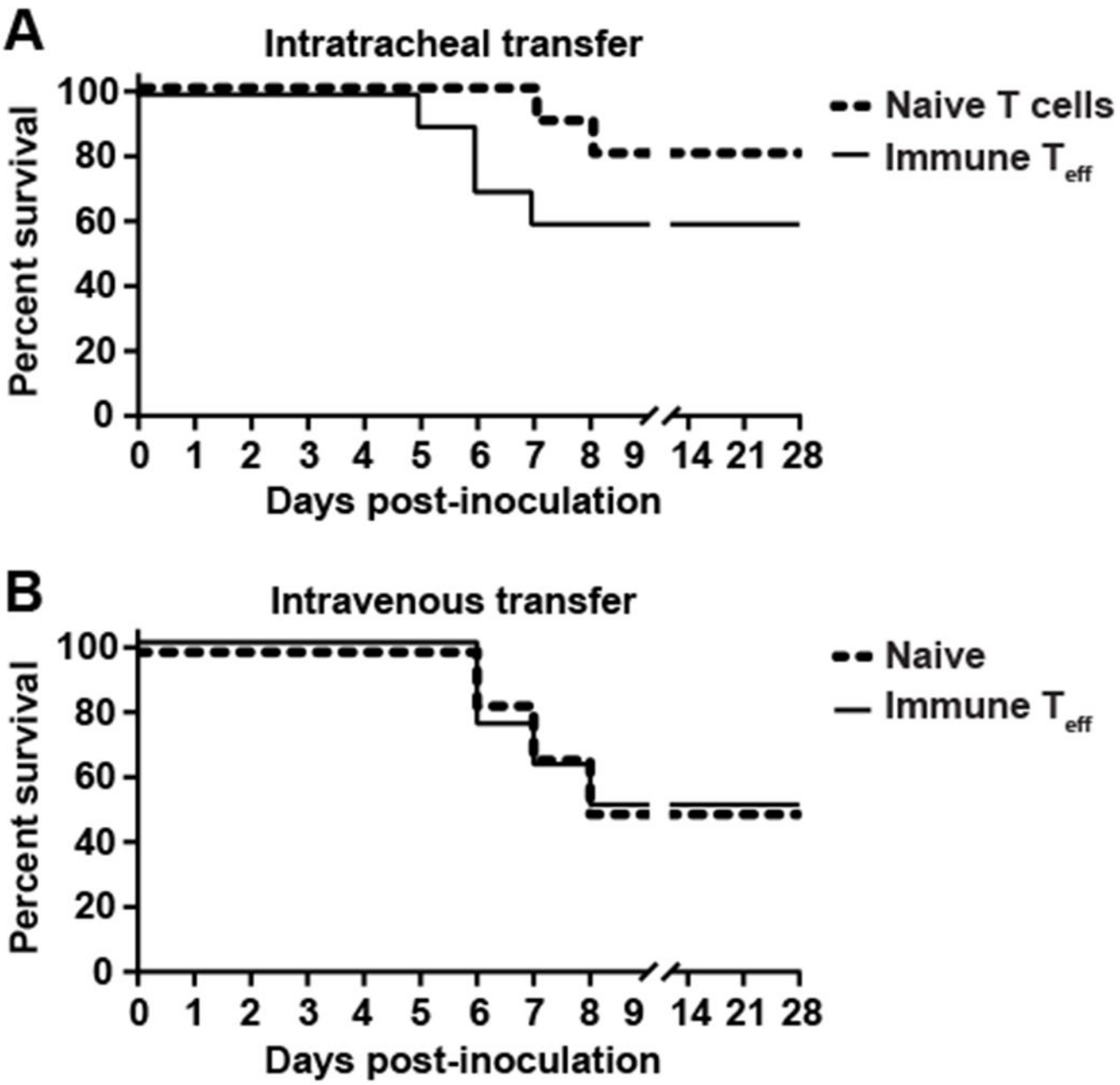

Figure 4. Adoptive transfer of pulmonary T cells does not protect against SARS-CoV-2 challenge.

A) Total T cells from naïve hACE2 mice or total T_{eff} from hACE2 immune animals were purified and $3.5x10^5$ cells were intratracheally transferred into naïve hACE2 animals or B) $1.4x10^6$ total T_{eff} from hACE2 immune animals were intravenously transferred into naïve hACE2 animals. One day later, mice were intranasally challenged with 10^2 PFU of SARS-CoV-2 and monitored for signs of illness. n=10 mice/group (A) or n=6-8 mice/group (B) and are combined from 2 independent experiments. Statistically significant differences

in survival between groups was determined using a log-rank (Mantel-Cox) test and found not to be statistically significant.