

# Patients with coronary artery disease have suppressed antiviral immunity due to hyperactivity of the immunological checkpoint CD155

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### ABSTRACT

Weak anti-viral immunity is associated with pre-existing cardiovascular illness, but the underlying mechanisms are yet unknown. In the current study, we show that Macrophages (M) in patients with Coronary Artery Disease (CAD) actively suppress the induction of helper T cells that are reactive to two viral antigens: the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein and the Epstein-Barr Virus (EBV) glycoprotein 350. The methyltransferase METTL3 was overexpressed by CAD M, which ca-

used N6-methyladenosine (m6A) to build up in the Polio Virus Receptor (PVR) mRNA. The PVR mRNA's 3' untranslated region had m6A alterations at locations 1635 and 3103 that stabilised the transcript and improved surface expression of the PVR-encoded CD155. Due to the patients' M's high expression of the immunoinhibitory ligand CD155, CD4+ T cells expressing CD96 and/or TIGIT receptors received unfavourable signals from the M. Reduced anti-viral T cell responses in vitro and in vivo were caused by METTL3hiCD155hi M's compromised antigen-presenting activity. The immunosuppressive M phenotype was brought on by low-density lipoprotein and its oxidised form.

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### INTRODUCTION

Strong risk factors for severe viral disease, which is worsened by high morbidity and mortality rates, include pre-existing cardiovascular conditions such as hypertension, CAD, cardiac arrhythmias, and congestive heart failure. Additionally, those with cardiovascular comorbidities don't react to immunizations as well as they should. A history of CAD was related with severe symptoms during the recent SARS-CoV-2 epidemic, illustrative of the poor anti-viral immunity in patients with CAD. Varicella zoster virus causes inadequate immune responses in CAD patients, and chronic EBV infection has been linked to cardiovascular disease. The recent pandemic has demonstrated that a better understanding of protective immunity is required to guide the therapeutic management of virally infected patients with pre-existing cardiovascular disease, even though the relationship between viral immunity and the progression of atherosclerotic disease is still insufficiently understood. The development of adaptive immunity, specifically the priming and proliferation of CD4+ T cells that support antibody-producing B cells

and virus-specific CD8+ killer T cells, is essential for protection against and clearance of viral infections. Each Coronavirus Disease 2019 (COVID-19) convalescent patient has peripheral blood that has been found to include SARS-CoV-2-specific CD4+ T cells<sup>8</sup>. Infected individuals with COVID-19 possess CD4+ T lymphocytes that are specific for the viral spike and nucleocapsid antigens. Such CD4+ T cells are present in some people who test negative for SARS-CoV-2, and they were likely stimulated by the common human coronaviruses that infect children and adults with upper and lower respiratory tract diseases. Similar to this, CD4+ T lymphocytes play a crucial role in defending the host against the harmful effects of EBV infection. The innate and adaptive immune systems of CAD patients are aberrant. T cells and M cells were shown to be the predominant tissue-resident cell types (10% M cells and 65% T cells, the majority of which were CD4+ T cells) in transcriptomic and cytometric single-cell study of atherosclerotic plaque lesions. Although it is unclear exactly how CD4+ T cells contribute to the development and maintenance of atherosclerosis, individuals with CAD have enlarged clonotypes of

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IFN-high-producing CD4<sup>+</sup>CD28 T cells. The integrity of the vascular system is threatened by these CD4<sup>+</sup> T cells' cytotoxicity toward endothelial cells. In addition to their function as lipid-uptaken effector cells and tissue-destructive effector cells, M cells play a crucial role in the activation of adaptive immunity as antigen-presenting cells. M from CAD patients impair antiviral T cell immunity because of abnormal co-inhibitory ligand PD-L1 expression. It is unknown if this deficiency is relevant to COVID-19 infection and persistent EBV infection. M expresses a variety of co-stimulatory and co-inhibitory ligands that affect communication with interacting T cells, similar to other expert antigen-presenting cells. Delivering activating and suppressive signals, M regulates the balance of T cell activation, tolerance, and immunopathology, with PD-L1 and the poliovirus receptor instructing T cells to stop their activation programme. Commonly found on monocytes, M, and myeloid dendritic cells, CD155 is a transmembrane glycoprotein from the nectin-like family of proteins that binds to three receptors on the surface of T cells and natural killer cells to transmit a stop signal. These receptors are TIGIT (T cell immunoreceptor with Ig and ITIM domains), CD96, and CD226. The high levels of CD155 expression in tumour cells encourage immune-evasive tactics and suggest that CD155 inhibition may be useful in anti-tumor immunotherapy. The balance of stimulatory and inhibitory ligands on antigen-presenting cells, which is regulated transcriptionally or post-transcriptionally, ultimately determines the degree of T cell activation. Significant post-transcriptional events affecting gene expression are known as mRNA modifications. The most common reversible alteration of mRNA, m6A controls translation, alternative splicing, and transcript stability. M6A is important for several cell types and is regulated by a collection of regulatory proteins that are further classified into "writer," "reader," and "eraser" proteins. The *METTL3*/*METTL14*/*WTAP* complex adds a methyl group to position N6 of adenosine to produce m6A. Lack of *METTL3* in mice causes embryonic mortality. Tumor invasion and proliferation are under the control of the *METTL3*-mediated m6A alteration. *METTL3* is important for remodelling and hypertrophy of cardiomyocytes in the cardiovascular system. By methylating *STAT1* mRNA25, *METTL3* encourages macrophage polarisation toward the pro-inflammatory M1 subtype and enhances dendritic cell maturation. Patients with CAD were unable to elicit CD4<sup>+</sup> T cell responses against the SARS-CoV-2 and EBV antigens, which is a requirement for efficient and sterile host defence. The incapacity of patients with CAD to expand anti-SARS-CoV-2-reactive T cells was not remedied by COVID-19 immunization. The immunological deficiency resulting from M's ineffective presentation of viral antigens and CD155's improper expression (encoded by PVR). By binding to the inhibitory receptors TIGIT and CD96 on memory CD4<sup>+</sup> T cells, CD155hi antigen-presenting cells reduced the activation of adaptive immunity. Extra CD155 expression resulted from prolonged mRNA stability, which was caused by the highly active methyltransferase *METTL3* and m6A-modified PVR mRNA enrichment. The responsiveness of CD4<sup>+</sup> T lymphocytes to viral antigens was restored by CD155-blocking antibodies and Small Interfering RNA (siRNA)-mediated inhibition of PVR and *METTL3*. Early on in the life cycle of monocytes and macrophages, *METTL3*hi expression was seen. The findings clarify m6A editing as a rate-limiting step in the production of protective immunity and characterise antigen-presenting M as essential effectors in anti-viral immunity. They also mechanistically link host protection to RNA epigenetics. For better management of viral infection in high-risk people, targeting m6A regulators to regulate the CD155 immunological checkpoint shows potential.

## RESULTS

### Patients with CAD fail to generate anti-viral T cell responses

To test the capacity of CAD patients and age-matched healthy controls to produce SARS-CoV-2-specific and EBV-specific T cells, we created an ex vivo assay system. The two main SARS-CoV-2 antigens, SARS-CoV-2 Spike (S) and Nucleocapsid (N) proteins, were pumped into Peripheral Blood Mononuclear Cells (PBMCs) from patients and controls. EBV glycoprotein gp350, the most prevalent glycoprotein expressed on the EBV membrane and the primary target for neutralising antibodies, was used to stimulate PBMCs concurrently. By observing the buildup of secreted IFN- $\gamma$ , we evaluated the robustness of antiviral T cell responses. In cultures from healthy people, SARS-CoV-2-induced IFN- $\gamma$  concentrations averaged 98 pg/ml<sup>1</sup>

, but patients with CAD only produced 43 pg ml<sup>-1</sup>. Additionally, flow cytometry was used to measure antigen-reactive T cells that are identified by the co-expression of CD69 and CD40L27. In contrast to individuals with CAD, who had only half the number of CD3<sup>+</sup>CD69<sup>+</sup>CD40L<sup>+</sup> T cells on day 5 after antigen stimulation, 0.77% of healthy T cells exhibited this characteristic. The attenuated response of individuals with CAD was attributed to the CD4<sup>+</sup> subset based on a comparison of CD69<sup>+</sup>CD40L<sup>+</sup> frequencies within the CD4<sup>+</sup> and CD8<sup>+</sup> subpopulations. Patients with CAD and healthy controls had comparable distributions of CD4<sup>+</sup> and CD8<sup>+</sup> T cell subtypes in newly extracted cell populations. The memory profile of T cells that responded to SARS-CoV-2 antigens is consistent with priming during infection with similar coronaviruses. Only spike protein stimulation resulted in spike-antigen-reactive T cell frequencies that were twice as high as nucleocapsid-reactive T cell frequencies. The mRNA COVID-19 vaccines from Moderna (mRNA-1273) and Pfizer-BioNTech (BNT162b2) produce detectable T cell responses to spike protein. We enlisted completely immunised healthy controls and patients with CAD in order to investigate if COVID-19 vaccination can help these individuals develop sufficient T cell immunity against SARS-CoV-2 antigens. Surprisingly, healthy persons who had received the vaccine produced twice as much IFN- $\gamma$  than those who had not. Additionally, spike-reactive CD69<sup>+</sup>CD40L<sup>+</sup> T cells reached 3% of CD4<sup>+</sup> T cells, which is three times greater than the 1% frequencies observed in healthy non-vaccinated donors. The frequency of anti-SARS-CoV-2-reactive T cells was not significantly altered in individuals with CAD after vaccination with the mRNA-based vaccines. Production of IFN- $\gamma$  was somewhat, but not significantly, increased by vaccination. 17 of 20 post-vaccination patients with CAD in the group had spike-induced CD69<sup>+</sup>CD40L<sup>+</sup> T cell frequencies of less than 1%. These findings collectively revealed a pool of IFN-producing CD4<sup>+</sup> memory T cells that expanded in response to viral antigens. Antiviral CD4<sup>+</sup> T cells were ineffective against SARS-CoV-2 and EBV antigens in patients with CAD, and vaccination with an mRNA-based vaccine was unable to correct the problem.

### CAD M $\phi$ overexpress the immunoinhibitory ligand CD155

The co-stimulatory and co-inhibitory signals produced by the antigen-presenting cell have a significant impact on the strength and longevity of antigen-specific T cell responses, which are dependent on antigen recognition. We identified 12 co-stimulatory and co-inhibitory compounds in the transcriptome of M obtained from patients and controls. Significant increases in PD-L1 and PVR transcripts were seen in CAD M, while PVR showed the greatest difference. A flow cytometric examination of controls and CAD M indicated that CD155 was highly expressed on the cell surface. Confocal imaging

of CD155 revealed that in CAD M, the protein was highly expressed both in the cytoplasm and on the cell surface. We looked at the *CD155<sup>hi</sup>* phenotype of M found within the atherosclerotic plaque. In atheroma tissue, dual-color immunohistochemistry identified *CD155* only on CD68<sup>+</sup> M, who live in plaques are a diverse group of people.

## METHODS

### Test for in vitro antigen presentation

In RPMI 1640 medium supplemented with 10% FBS, PBMCs were primed with viral antigens for 5 days. Antigen-stimulated PMBCs were washed on day 5 and stored in antigen-free media for 24 hours to eliminate the antigens in order to test for recall reactions. On day 6, syngeneic macrophages that had been loaded with antigen by overnight culture were combined with primed PBMCs. T cell activation was assessed six hours later using flow cytometry to detect the surface receptors CD69 and CD40L.

### Monocyte priming

Healthy people's PMBCs were used to isolate CD14<sup>+</sup> monocytes using the EasySep Human Monocyte Isolation Kit (STEMCELL Technologies, 19359). Monocytes were raised in media that also contained 10% plasma from human donors with levels of TGs, LDL, and HDL that were known, comprehensive lipid profiles. The three categories of plasma samples were TG-high, TG-low plus LDL-low, and TG-low plus LDL-high.

### Immunofluorescence and confocal microscopy

Dual-color immunostaining procedures were previously discussed. In glass-bottom tissue culture plates, cells were fixed with a 4% paraformaldehyde solution (Affymetrix) before being treated with a primary antibody at 4°C for an overnight period and a fluorescence-conjugated secondary antibody at room temperature for two hours. Atherosclerotic plaques were cut into 4-mm-thick sections for tissue staining, and the sections were permeabilized with 0.5% Triton X-100 in PBS for 20 minutes. Primary antibodies were incubated on tissue sections for an overnight period at 4°C, and secondary antibodies were incubated for an hour at 37°C. DAPI (Santa Cruz Biotechnology) was used to mark nuclei for 10 minutes at room temperature. The All-in-One Fluorescence Microscope BZX800E or the Olympus fluorescence microscope equipment were used to analyse the images.

### Western blotting

Techniques used for immunoblotting have been described previously. In essence, cells were collected and then lysed in RIPA buffer with proteinase inhibitor. Proteins were transferred to PVDF membranes after being electrophoresed in 4%-15% SDS-PAGE. The membranes were treated with the primary antibody anti-METTL3 (E3F2A) rabbit monoclonal antibody at 4°C for overnight and the secondary antibody anti-rabbit IgG HRP-linked antibody at room temperature for 1 hour following a 1-hour blocking step in 2% BSA.