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Early View

Research Letter

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SARS-CoV-2 (E)-protein induces rapid TLR2-mediated T cell activation in mouse lungs revealed by intravital lung microscopy

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To the editor:

The SARS-CoV-2 envelope (E)-protein is a conserved structural protein of the corona virus family with a key role in SARS-CoV2 pathogenicity [1]. Its sequence conservation among different SARS-CoV-2 viral variants and related coronaviruses makes it a promising candidate for preventive and therapeutic strategies [2]. (E)-protein acts as a viroporin that disrupts the host cell plasma membrane and triggers cell death [3]. It also mediates SARS-CoV2 sensing and inflammatory signalling via activation of the TLR2 receptor in macrophages [4, 5] driving innate immune cell activation and cytokine storm-induced mortality in patients with COVID-19. Cytokines and T cell receptor (TCR) cross-reactivity are described to cause TLR2-dependent activation of T cells, termed innate or bystander activation, challenging the dogma of exclusive adaptive antigen-specific T cell activation [6–8]. If SARS-CoV2 (E)-protein can activate innate T cell function and whether TLR2 signaling is involved is currently unknown.

Here, we combined *in vivo* labelling of T cells with intravital lung microscopy (IVM) to investigate the capacity of SARS-CoV-2 (E)-protein in T cell activation. IVM is a unique imaging approach that monitors the dynamics of cell behavior in real-time, without the need for tissue extraction [9]. Mice were ventilated with oxygen via intratracheal intubation, and an intercostal window was surgically implanted to expose the left lung lobe. To stabilize the lung for fluorescence microscopy, negative pressure was applied by vacuum suction (~20 mm Hg) via the window to bring the tissue into contact with the cover glass [10]. Lung-IVM was performed 4 or 24 hours after intratracheal instillation of recombinant (E)-protein. To visualize circulating lymphocytes, $CD8^+$ and $CD4^+$ T cells were labeled by intravenous injection of fluorescent antibodies 20 minutes before imaging (Fig. 1A). This *in vivo* labelling approach uncovered acute infiltration of $CD8^+$ T cells from the vasculature into the alveolar region of the lung induced by (E)-protein. T cell recruitment in alveolar microvessels and subsequent infiltration was evident as early as 4 hours post instillation and remained detectable at 24 hours (Fig. 1B), as also confirmed by flow cytometry (Fig. 1C). Notably, no significant recruitment of $CD4^+$ T cells was observed (Fig. 1B). Detailed time-lapse analysis of the $CD8^+$ T cell dynamic revealed a prominent decline in migration velocity following (E)-protein instillation (Fig. 1D). While control group T cells exhibited transient contacts with

microvascular walls ("vessel-tethering behavior"), E-protein treatment induced increasing numbers of T cells to maintain prolonged contacts. Specifically, the majority of these $CD8^+$ T cells transitioned to a crawling phenotype within the alveolar region, a behavior that persisted for up to 24 hours (Fig. 1D). Immunohistology of lung tissue co-stained for $CD8^+$ T cells and MHC class II^+ antigen presenting cells (APC) revealed the accumulation and aggregation of T cells and APCs in nodular inflammatory foci (NIF) enriched at perivascular regions and around large airways of the lung in (E)-protein challenged mice compared to control tissue (Fig. 1E). Such NIFs, characterized as organized clusters of APCs and T cells that form at sites of infection or inflammation, e.g. in the lung, are thought to orchestrate local immune responses [11]. In addition, we observed that $CD8^+$ T cells within these NIFs prominently expressed the innate activation marker NKG2D and the cytotoxic effector molecule granzyme B (Fig. 1F). Taken together, our lung-IVM and immunohistochemical findings suggest that pulmonary delivery of SARS-CoV2 (E)-protein triggers rapid recruitment of $CD8^+$ T cells into the lungs followed by their assembly into localized inflammatory structures and differentiation into activated cytotoxic effector T cells (Fig. 1F). As these mice were not previously exposed to SARS-CoV2 (E)-protein, we hypothesized that this rapid $CD8^+$ T cell activation might occur through a mechanism akin to bystander activation. To explore this, we isolated $CD8^+$ T cells from splenocytes of naïve mice and stimulated them with (E)-protein, and in parallel with cytokines and TCR agonists (αCD3/αCD28 antibodies). (E)-protein together with cytokines or TCR agonists significantly upregulated the expression of CD69 on $CD8^+$ T cells but was ineffective by itself (Fig. 1G-H). In contrast to αCD3/αCD28 antibodies, co-application of cytokines and (E)-protein also effectively stimulated secretion of interferon (IFN)γ (Fig. 1I) suggesting that TCR activation is dispensable for (E)-protein effects. Stimulatory effects were completely absent in $CD8^+$ T cells harboring a deletion of TLR2 ($Tlr2^{-/-}$) (Fig. 1G-I). Effects of Pam3CSK4, which is a known TLR2 agonist, were also abolished in these $Tlr2^{-/-}$ $CD8^+$ T cells.

The TLR2-mediated innate activation of $CD8^+$ T cells was further corroborated *in vivo* by lung-IVM in $Tlr2^{-/-}$ mice. (E)-protein was effectively delivered into the lungs both in WT and $Tlr2^{-/-}$ mice as evidenced by strong infiltration of fluorescently labeled neutrophils in both mouse strains (Fig. 1J). Deficiency of Tlr2, however, completely prevented early (E)-protein-induced infiltration of $CD8^+$ T cells into the alveolar region (Fig. 1J).

Antigen-specific and TCR-dependent $CD8^+$ T cell responses are crucial for effective immune responses against SARS-CoV-2 infection. They are not only essential for viral clearance but also provide memory and cross-protection from viral variants [12]. The differentiation of antigen-specific adaptive T effector cells is commonly assumed to require several days following antigen- and TCR-mediated priming. In a proof-of-concept mouse study, we here discover an early TLR2-mediated recruitment and activation of $CD8^+$ T cells into cytotoxic T cells by the SARS-CoV2 envelope protein within 4-24 hours after (E)-protein challenge *in vivo* using intravital microscopy of mouse lungs [10]. Our data suggest TCR-independent bystander activation of $CD8^+$ T cells as shown for immunologically naïve T cells with a memory-like phenotype [13]. Our proof-of-concept findings consistently support the conclusion that (E)-protein can modulate $CD8^+$ T cell responses during viral infection, highlighting potentially clinically relevant aspect of anti-viral response. Limitations of our study include the high and probably unphysiological concentration of recombinant (E)-protein as well the lack of a specific mechanism how $CD8^+$ T cell are activated by (E)-protein *in vivo*. We speculate that this involves cytokine signalling via acute neutrophil and macrophage activation.

Early innate bystander $CD8^+$ T cell activation has also observed in SARS-CoV2 infected humans correlating with disease resolution and the prevention of clinical progression to severe disease [15]. We concur with Karl et al. who suggested that "…bystander-activated T cells may play an important role in the early viral defense" [16]. While TLR2 activation of macrophages has been linked to COVID-19 severity [5] with TLR2 antagonists demonstrating protective and adjuvant effects [4, 5], early activation of $CD8^+$ T cells is conversely associated with protection against severe disease [14]. Further research is needed to harness our observation of a rapid SARS-CoV-2 E-protein–induced $CD8^+$ T cell response for diagnostic and therapeutic applications.

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Figure legend

Fig. 1: Intravital lung microscopy (lung-IVM) of T cells reveals fast recruitment and activation of $CD8^+$ T cells upon challenge with SARS-CoV2 (E)-protein.

A) Experimental scheme: C57Bl/6J mice (100-200 days old) were instilled with 25 μg/mouse (E)-protein and investigated after 4 or 24 hours (hr). T cells were visualized via intravenous injection of fluorescently labelled antibodies against CD8 or CD4 (αCD8-APC, 3 µg/mouse, clone 53-6.7, αCD4-PE, 2 µg/mouse, clone GK1.5, both BD Pharmingen™) 20 minutes (min) before performing lung-IVM. Granulocytes were labelled using αLy6G-AF488 (3 µg/mouse, clone 1A8, BioLegend). Experiments were performed according to the guidelines of the Regierung von Oberbayern (District Government of Upper Bavaria) under the approval number ROB-55.2Vet-2532.Vet_02-19-150.

B) Representative images taken from lung-IVM time lapse videos (duration 10 min) are shown with $CD8^+$ T cells labeled in green and $CD4^+$ T cells labeled in red after 4 and 24 hr after (E)-protein instillation. $CD8^+$ and $CD4^+$ T cells were quantified per Field of View (FOV, 0.16 mm^2) using the mean of 5 FOV/mouse (n=4 mice/group). Contrast rich roundish structures in the lung tissue, outlined by tissue autofluorescence in the green channel, are alveoli, surrounded by microvessels. Unpaired t-test was applied using the respective experimental control with *p<0.001 (***)* and *p<0.01 (**).*

C) Flow cytometry analysis to determine percentage of $CD3^+CD8^+$ T cells of $CD45^+$ cells in lungs of mice that had been instilled with (E)-protein 4 hr before confirming elevated numbers of $CD8^+$ T cells in the lungs. Unpaired t-test was applied with *p<0.05 (*).*

D) Average track velocity and classification of $CD8^+$ T cell behavior into crawling, tethering or adherent $CD8^+$ T cells in control mice and mice instilled with (E)-protein 4 or 24 hr before. Each data point represents an individual cell. Adherent cells were defined as cells that showed no displacement during the 20 min of imaging, crawling cells are T cells with an average velocity of less than 20 µm/min, while tethering cells are defined as cells that jump fast from one point to the next within the Field of View (FOV, 0.16 mm^2) using Fiji Image J software (TrackMate plugin). One-way ANOVA with Dunnett's multiple comparison testing was applied with *p<0.0001 (****).* For velocity measurements, 500-1000 cells were analyzed, behavior classification was done with 100 $CD8^+$ T cells in total per condition.

E) Representative whole lung sections showing nodular inflammatory foci (NIFs) in lungs of mice that had been intratracheally instilled with (E)-protein 24 hr before. Lungs were fixed,

paraffin-embedded and stained for CD8 (αCD8 rabbit α-monoclonal antibody, EPR20305, Abcam, dilution 1:500, EDTA antigen retrieval buffer) and MHC class II (α-MHC II rabbit polyclonal antibody, PA5-116876, Thermo Fischer Scientific) to identify $CD8^+$ T and antigen-presenting cells, respectively, with DAPI (Sigma Aldrich) as a nuclear stain to detect all cells. Bar represents 50 μm.

F) Quantification of immunofluorescence staining of $CD8^+$ T cells expressing NKG2D (αNKG2D rabbit polyclonal antibody, PA5-97904, Thermo Fischer Scientific) or Granzyme B (αGzmB rabbit monoclonal antibody, D6E9W, Thermo Fischer Scientific) in paraffin-embedded lungs of mice that had been intratracheally instilled with (E)-protein 24 hr before. $CD8^+$ $NKG2D^+$ and $CD8^+$ $GzmB^+$ T cells were quantified per Field of View (FOV, 0.233 mm^2). 5 FOV/mice were counted, and the mean was calculated for each mouse (n=4/group). Unpaired t-test was applied with *p<0.0001 (****).*

G) Stimulation of WT and $Tlr2^{-/-}$ $CD8^+$ T cells *in vitro*. $CD8^+$ T cells were isolated from spleens using mouse $CD8\alpha^+$ T Cell Isolation Kit (Miltenyi Biotec) and the autoMACS Pro separator. WT $CD8^+$ T cells were cultivated either without any stimulation (control) or stimulated with (E)-protein (2 μg/ml, Thermo Fisher Scientific), Pam3CSK4 (10 ng/ml, EMC microcollections), cytokines (interleukin-2 (IL-2, 20 ng/ml, Biotest) and IL-15 (5 ng/ml R&D Systems)) or by plate-coated antibodies αCD3 (1 μg/ml, clone 145-2C11, eBioscience) and αCD28 (5 μg/ml, clone 37.51, BD Pharmingen™) at 37°C, 5% CO_2 for 16 hr. T cell activation was determined by detecting CD69 expression on $CD8^+$T cells using MACSQuant Analyzer 16 (Miltenyi Biotec) and is shown as percentage of $CD69^+$ $CD8^+$ T cells (αCD8-53-6.7, αCD69-H1.2F3, both from BioLegend). Pair-wise significance was calculated using Two-way ANOVA with Tukey's multiple comparison testing, *p<0.05 (**).

H) Co-stimulatory effects of (E)-protein and Pam3CSK4 on purified $CD8^+$T cells. T cell activation was quantified by CD69 expression on $CD8^+$ T cells upon cytokine (IL-2/IL-15) or TCR stimulation (αCD3/αCD28). A representative flow cytometry analysis is shown with quantification of CD69 expression as fold activation over the respective control after background subtraction (WT: n=8; $Tlr2^{-}/^{-}$: n=9). Pair-wise significance was calculated using Two-way ANOVA with Tukey's multiple comparison testing, *p<0.01 (**)* and *p<0.001 (***)*. We thank Carsten Kirschning for generously providing the $Tlr2^{-/-}$ mice. (*$Tlr2^{tm1Kir}$*).

I) Supernatants of stimulated WT and Tlr2$^{-/-}$ CD8^{+} T were analyzed for interferon (IFN)γ using the U-PLEX Mouse IFNγ Assay from MSD (Meso Scale Discovery) and is shown in log scale. Significance was calculated using the ratio paired t-test with *p<0.05 (*), p<0.01 (**)* and *p=0.0001 (***)*.

J) Representative images taken from lung-IVM time lapse videos are shown with CD8^{+} T cells labeled in green, CD4^{+} T cell labeled in red, and neutrophils detected by Ly6G labeling in purple. *C57Bl/6J* WT or *Tlr2*$^{-/-}$ mice were intratracheally instilled with or without (E)-proteins 4 hr before and intravenously injected with fluorescently labelled antibodies (αLy6G-AF 488, 3 µg/mouse, clone 1A8, BioLegend; αCD4-PE, 2 µg/mouse, BD Pharmingen™; αCD8-APC, 3 µg/mouse, BD Pharmingen™) 20 min before performing thoracic surgery, vacuum stabilization of the lungs and fluorescence microscopy. CD8^{+} and CD4^{+} T cells and Ly6G^{+} neutrophils were quantified per Field of View (FOV, 0.16 mm^{2}) using the mean of 5 FOV/mouse (n=4 mice/group). One-way ANOVA with Dunnett's multiple comparisons test was applied with *p<0.01 (**), p<0.0001 (***).*

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