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Letter

Are NKT cells a useful predictor of COVID-19 severity?

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The article by (Kreutmair et al., 2021), published online with May 9, 2021 issue of *Immunity*, delineated COVID-19-related immune signatures between mild and severe COVID-19 hospital admissions, and other hospital-acquired pneumonia. This timely study includes highly relevant findings for the current pandemic. One important observation in this study, that circulating natural killer T (NKT) cell frequency is a powerful predictive biomarker for severe COVID-19, was of particular interest to us. The message is that where NKT cells fall below 2.3% of patient peripheral blood T cells within 1–2 days of hospital admission, development of severe disease is strongly predicted. That the frequency of NKT cells in patient blood might so accurately predict the course of disease has obvious clinical implications.

Notably, this observation aligns with another report in which NKT cells were shown to be reduced in patients with severe disease (Zingaropoli et al., 2021) but is inconsistent with Zhang et al. (2020) and Mazzoni et al. (2020). In these studies, NKT cells were defined as $CD3^+$ $CD56^+$ cells (Kreutmair et al., 2021; Mazzoni et al., 2020; Zhang et al., 2020; Zingaropoli et al., 2021). While this classification, or variations thereof such as NK/T cells or NKT-like cells, is sometimes used in clinical analyses; to most immunologists the term NKT cell refers to lipid antigen-reactive CD1d-restricted T cells, most of which do not express CD56 (Le Dieu et al., 2007). These cells express a semi-invariant T cell receptor (TCR), comprising an invariant TCR-α chain pairing TRAV10 and TRAJ18 genes (Vα24-Jα18) and a constrained array of TCR-β chains enriched for TRBV25-1 (Vβ11). CD1d-based intrathymic selection engenders a divergent developmental pathway for NKT cells relative to peptide-MHC-restricted T cells, and this imbues an innate-like phenotype and functional capacity, including expression of the NK lineage marker (CD161c in humans or NK1.1 in mice) which originally precipitated the term "NKT cells." In humans, NKT cells, defined using CD1d tetramers loaded with the archetypal lipid antigen α-galactosylceramide (αGalCer), are typically less than 0.1% of blood T cells (Le Dieu et al., 2007; Lee et al., 2002). Thus, while the finding that $CD3^+$ $CD56^+$ T cells are predictors of COVID-19 severity (Kreutmair et al., 2021) is intriguing, the vast majority of these cells are highly unlikely to be CD1d-restricted NKT cells. Instead, $CD3^+$ $CD56^+$ cells are heterogeneous, including various 'unconventional' non-MHC-restricted T cells such as mucosal-associated invariant T (MAIT) cells and $\gamma\delta$ T cells, that outnumber NKT cells by 10-100-fold (Godfrey et al., 2015), as well as other T cells that express CD56 such as $CD8^+$ T cells associated with cytotoxicity, reviewed in Van Acker et al., (2017). Accordingly, further dissection of this population could provide important insight into the immunological processes associated with severe COVID.

Although the $CD3^+$ $CD56^+$ phenotype does not necessarily define a particular population of unconventional T cells, it is possible that changes in some, or all, of these populations of MAIT cells, $\gamma\delta$ T cells, and NKT cells are impacting on the frequency of $CD3^+$ $CD56^+$ cells in patients with COVID-19. Indeed, other studies have suggested that these cells may be selectively depleted in the blood and traffic to the lungs during COVID-19 disease (Jouan et al., 2020; Parrot et al., 2020). Similar observations have been observed for unconventional T cells in other disease settings (Godfrey et al., 2015). Although viruses do not furnish the lipid or riboflavin-metabolite antigens or phosphoantigens that activate NKT cells, MAIT cells, and $\gamma\delta$ T cells, respectively, these cells can react to pathogens in a TCR-independent manner via cytokines such as IL-12 and IL-18 that can be more prevalent during viral infection (Godfrey et al., 2015).

To clarify the proportion of NKT, MAIT, and $\gamma\delta$ T cells within $CD3^+$ $CD56^+$ cells of individuals with COVID-19, we examined peripheral blood mononuclear cells (PBMCs) derived from a hospitalized patient cohort with CD1d-α-GalCer tetramer, MR1-5-OP-RU tetramer, and anti- $\gamma\delta$ TCR mAb to define NKT cells, MAIT cells, and $\gamma\delta$ T cells, respectively, via flow cytometry (Figures S1A and S1B). This cohort includes COVID-19 patients that were admitted to a hospital ward (COVID Ward, moderate disease), COVID-19

patients admitted into the intensive care unit (COVID ICU, severe disease), or non-COVID-19 patients in the intensive care unit ($COVID^{neg}$ ICU). As anticipated, we show that within COVID Ward and COVID ICU patients, or healthy PBMC controls, $CD56^+$ T cells were largely composed of γδ T cells and MAIT cells, whereas NKT cells make up <1% of this population (Figures S1A and S1B). We show in healthy donor samples that many MAIT cells and γδ T cells, and most NKT cells, do not express CD56 (Figures S1C and S1D). Furthermore, focusing on these cell types, we failed to detect any clear differences in the frequencies of $CD56^+$ MAIT cells, γδ T cells, and NKT cells (although only 5 ICU samples were tested for NKT cells) between COVID Ward and COVID ICU patients (Figures S1A and S1B).

We also examined the entire population of $CD3^+$ $CD56^+$ cells in samples taken from patients within 2 days of hospital admission (Figure S1E) and observed no significant differences in the frequency of this population between COVID Ward, COVID ICU, $COVID^{neg}$ ICU patients, or healthy donors within our cohort. In our hands, the mean frequencies of $CD56^+$ cells within the $CD3^+$ T cell populations of these groups were 10.3%, 8.7%, 14.5%, and 9.6%, respectively (Figure S1E). We also failed to detect differences in the frequency of γδ T cells or MAIT cells between COVID Ward, COVID ICU, and $COVID^{neg}$ ICU patients (Figure S1F), apart from a significant decrease of circulating MAIT cell frequencies in all hospitalized patient groups compared with healthy donors, consistent with other studies (Jouan et al., 2020; Parrot et al., 2020). An important caveat is that our sample size is lower than that of the Kreutmair et al. (2021) and Zingaropoli et al. (2021) studies, so the discrepancy in our findings regarding the frequency of $CD3^+$ $CD56^+$ T cells should be taken with caution, although our findings do align with other studies (Zhang et al., 2020; Mazzoni et al., 2020).

While it could be argued that the concerns raised in this letter boil down to semantics, the use of surrogate phenotypes such as $CD3^+$ $CD56^+$ T cells to define NKT cells may lead to confusion when ascribing roles to these cells in health and disease. For example, in discussing the significance of these cells (Kreutmair et al., 2021; Zingaropoli et al., 2021), references were made to the roles they may play in influenza or contributing IL-4 to form germinal centers for enhancing antibody production based on studies that were actually referring to CD1d-restricted NKT cells, which, as we have established, are not the same as $CD3^+$ $CD56^+$ T cells. This may also spark problems when others look for NKT cells in COVID-19 patients using different approaches such as CD1d-α-GalCer tetramer or TRAV10 and TRBV25-1 co-staining, which are commonly used approaches to define these cells. Moreover, innate-like T cells such as MAIT cells and γδ T cells within the $CD3^+$ $CD56^+$ subset are likely to have distinct and potentially opposing functions. The role of MAIT, NKT, and γδ T cells in SARS-CoV-2 infection is still unknown, but some reports suggest that they include lung-homing cells that exacerbate disease through IL-17 production in airways (Jouan et al., 2020; Parrot et al., 2020). Other studies have suggested a protective role for these cells in other (non-SARS-CoV-2) viral infections, reviewed in Godfrey et al. (2015). Thus, it is certainly important to gain a better understanding of these cells in COVID-19, using markers that accurately define them, and how they contribute to COVID-19 disease severity. CD1d and MR1 tetramers used to identify NKT cells and MAIT cells are available from the NIH tetramer facility and anti-γδ TCR antibodies are also readily available from commercial suppliers, and further studies using these approaches are eagerly awaited.

In conclusion, while there are many significant and important findings in the Kreutmair study (Kreutmair et al., 2021), we wish to clarify that: (1) $CD56^+$ T cells do not equate to NKT cells as most people understand them; (2) $CD56^+$ T cells include a heterogeneous collection of T cells, mostly made up of MAIT and γδ T cells, and (3) the use of $CD56^+$ T cells as biomarker to predict severe COVID-19 in hospital admissions represents an intriguing observation, but based on our smaller analysis, this requires further investigation and validation.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.immuni.2022.01.005.

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Experiments conformed to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice. Written informed consent was obtained from all donors prior to the study. The study was approved by the Austin Health (HREC/63201/Austin-2020) and the University of Melbourne (#2057366.1 and #2056901.1) Human Research Ethics Committees.

DECLARATION OF INTERESTS

D.I.G. and N.A.G. have a provisional patent application submitted that describes immunotherapy and vaccine approaches for COVID-19. All other authors declare no competing interests.

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