

# BNT162b2 COVID-19 vaccination elicits the expansion of CD16<sup>+</sup>CD8<sup>+</sup> T cells endowed with natural killer cell features



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**Background:** The Pfizer-BioNtech vaccine, also known as BNT162b2, was developed using a novel technology based on mRNA and protects against coronavirus disease 2019 (COVID-19) via induction of specific antibody and T-cell responses. Much less is known about the broader effects of this new class of vaccines on unconventional cellular components of the immune system.

**Objectives:** We aimed to characterize a subset of unconventional T cells emerging following BNT162b2 mRNA vaccination.

**Methods:** Peripheral blood from a total of 30 human healthy individuals who received 2 doses of the BNT162b2 mRNA vaccine was collected for the analysis of T-cell compartment by using multiparametric flow cytometry and single-cell transcriptome analyses.

**Results:** In the peripheral blood of individuals undergoing BNT162b2 vaccination, we observed a sizable fraction of CD8<sup>+</sup> T cells expressing CD16, a low-affinity FcR for IgG. These cells were severe acute respiratory coronavirus 2-specific, characterized by IFN- $\gamma$  response gene transcripts and stimulation through CD16 and other natural killer (NK)-cell innate receptors elicited a functional response. Both CD16 and NKp30 could be induced on NKp80<sup>+</sup> CD8<sup>+</sup> T cells and the engagement of NKp80 in combination with CD16 resulted in synergic effects. CD16<sup>+</sup> CD8<sup>+</sup> T cells also showed a high expression of the inhibitory

receptor G protein-coupled receptor 56 (GPR56), capable of limiting their activation via CD16.

**Conclusions:** These data indicate that BNT162b2 COVID-19 vaccination provides an additional large fraction of antibody-dependent cellular cytotoxicity (ADCC)-capable effector cells, endowed with innate functions and therefore able to potentially counteract a much wider array of diseases, including cancer. (J Allergy Clin Immunol 2025;155:1981-92.)

**Key words:** BNT162b2 mRNA vaccine, CD16/Fc $\gamma$ RIIIA, innate-like T cells, ADCC, NK cells, spike-specific CD8<sup>+</sup> T cells, human

The Pfizer-BioNtech (New York, NY) vaccine, also known as BNT162b2, was developed using a novel technology based on mRNA and demonstrated 95% efficacy against symptomatic coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).<sup>1,2</sup> Emerging understanding of the immune effector mechanisms of BNT162b2 vaccine has been so far mainly focused on adaptive immunity, such as the nature of antibodies and antigen-specific T-cell responses.<sup>3</sup> In spite of the proven clinical efficacy and widespread use of BNT162b2, a comprehensive understanding of the mechanisms by which BNT162b2 can elicit the different protective components of the immune responses has not yet been accomplished. By characterizing CD8<sup>+</sup> T cells from donors vaccinated with BNT162b2 vaccine, we observed a significant proportion of CD8<sup>+</sup> T cells expressing Fc $\gamma$ RIIIA (CD16), a low-affinity FcR for IgG characterized for its role in mediating antibody-dependent cellular cytotoxicity (ADCC).<sup>4</sup> The existence of T lymphocytes bearing FcRs for immunoglobulins has long been recognized, and growing evidence suggests the potential importance of this subset of T cells in mediating potent ADCC activity.<sup>5,6</sup> In particular, a subset of late-stage differentiated CD8<sup>+</sup> T cells acquiring ADCC activity has been observed in chronic infections that are difficult for the immune system to control, such as hepatitis C virus (HCV)<sup>7</sup> and human immunodeficiency virus (HIV).<sup>8</sup> Although HCV and HIV-1 differ in their mechanism of pathogenesis, in both viral infections, the expansion of CD8<sup>+</sup> T lymphocytes with natural killer (NK)-like functionality may represent a way for the immune system to take full advantage of the cytolytic program of CD8<sup>+</sup> effector T cells.<sup>7,8</sup> On the same line, CD16<sup>+</sup> T cells have been identified also in patients with severe COVID-19, although they displayed a less differentiated phenotype, not restricted only to CD8<sup>+</sup> T cells, that determines an excessive cytotoxicity induced by complement split product-rich inflammatory milieu.<sup>9</sup> In the context of COVID-19

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**Abbreviations used**

|             |                                                 |
|-------------|-------------------------------------------------|
| ADCC:       | Antibody-dependent cellular cytotoxicity        |
| COVID-19:   | Coronavirus disease 2019                        |
| CTX:        | Cetuximab                                       |
| EMRA:       | Effector memory re-expressing CD45RA            |
| GPR56:      | G protein-coupled receptor 56                   |
| GrzB:       | Granzyme B                                      |
| HCMV:       | Human cytomegalovirus                           |
| HCV:        | Hepatitis C virus                               |
| MAIT:       | Mucosa-associated invariant T                   |
| NK:         | Natural killer                                  |
| PMA+I:      | Phorbol 12-myristate 13-acetate and ionomycin   |
| SARS-CoV-2: | Severe acute respiratory syndrome coronavirus 2 |
| scRNA-seq:  | Single-cell RNA-sequencing                      |
| TCR:        | T-cell receptor                                 |

vaccination, recent findings have indicated that mucosa-associated invariant T (MAIT) cells, a subset of unconventional T cells with innate-like capacity, can play an important role in the initial priming events of the adaptive immune response to ChAdOx1 viral vector.<sup>10</sup> Also, positive associations have been observed between MAIT cell percentage and adaptive responses to Pfizer-BioNTech vaccine.<sup>11</sup> Here, we observed that a subset of CD8<sup>+</sup> T cells expressing CD16 was significantly increased on administration of BNT162b2 vaccine. This expanded CD16<sup>+</sup> CD8<sup>+</sup> T-cell subset displays a hybrid profile combining features of both CD8<sup>+</sup> T cells and NK cells. Remarkably, triggering of CD16 and other innate activating receptors evoke a potent response in these CD8<sup>+</sup> T cells, thus significantly contributing to the cytotoxic effector cell pool in vaccinees.

**METHODS****Study design**

A total of 30 individuals from the sanitary personnel and scientists operating at the University Hospital Policlinico G. Martino of Messina were enrolled for this study. Participants gave written informed consent according to the Declaration of Helsinki. Donor's whole blood and sera were collected at baseline (day 0), 1 week (day 28) and 6 months after boosting with the second dose of Pfizer-BioNTech BNT162b2 vaccine. Inclusion criteria comprised being older than 18 years, without immunocompromised disease or treatment, and no significant comorbidity. All donors were screened for SARS-CoV-2 by sera measurement of spike protein and nucleocapsid-specific IgG. Donors who showed high concentrations of antibodies against SARS-CoV-2 N-antigen at baseline were removed and few individuals (n = 4) were removed at later time points because they underwent COVID-19 in the course of the study. All patients enrolled in this study, at any time point, were negative for antibodies against HCV, hepatitis B virus (HBV), or HIV. Information on the donor's age, sex, race, cytomegalovirus status, and frequency of CD16<sup>+</sup>CD8<sup>+</sup> T cells are listed in Table E1 in this article's Online Repository (available at [www.jacionline.org](http://www.jacionline.org)).

**Cytotoxicity assay**

ADCC was performed as previously described.<sup>12</sup> The human NSCLC cell line A549 (CCL-185; ATCC, Manassas, Va) was

incubated with cetuximab (CTX 1 µg/mL) for 30 minutes at 37°C and washed twice with PBS. Target cells were cultured with volunteers' PBMCs collected at day 0, day 28, or 6 months following BNT162b2 vaccine administration at effector-to-target ratio (E:T) of 2:1. Effector cells cultured with only targets or CTX were used as controls. Anti-CD107a (1/100 dilution; Beckman Coulter, Indianapolis, Ind) was added at the beginning of culture. Following 6 hours of cytotoxic assay, cells were collected and stained for CD45, CD3, CD8, and CD56. The level of expression of CD107a was analyzed on both NK cells and CD8<sup>+</sup> T cells.

**In vitro stimulation of donor's PBMCs with BNT162b2, Nuvaxovid, and Vaxigrip Tetra vaccines**

PBMCs derived from 6 volunteers collected before vaccination (day 0) were stimulated for 7 days with 100 ng/mL, 500 ng/mL, or 1 µg/mL of mRNA-based BNT162b2 COVID-19 vaccine (Comirnaty, Pfizer-BioNTech),<sup>13</sup> with 10 µg/mL of quadrivalent inactivated strains from Vaxigrip Tetra 2023/2024 (Sanofi, Paris, France)<sup>14</sup> and with 500 ng/mL recombinant spike protein-based Nuvaxovid vaccine (NVX-CoV2373; Novavax, Gaithersburg, Md). Nuvaxovid dose (500 ng/mL) was chosen after performing a dose escalation assay (100 ng/mL, 500 ng/mL, 1 µg/mL, and 5 µg/mL) and evaluating, in both total leukocytes (CD45<sup>+</sup>) and CD8<sup>+</sup> T cells of PBMC donors, the frequency of viable and activated cells (Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Expression of CD16, NKp80, CD69, and intracellular granzyme B (GrzB)<sup>15,16</sup> was evaluated on CD8<sup>+</sup> T cells by *t*-distributed stochastic neighbor embedding analysis (*t*-SNE). Intracellular staining for SARS-CoV-2 antigen-specificity assay was performed.

**Detection of SARS-COV-2 specific T cells**

PBMCs of vaccinees were collected at day 0, day 28, and 6 months after BNT162b2 COVID-19 vaccination and restimulated for 24 hours with or without SARS-CoV-2 PepTivator Protein S pool (1 µg/ml of each peptide; Miltenyi Biotec, Bergisch Gladbach, Germany) as previously described.<sup>17</sup> Low dose of IL-2 (250 UI/mL) was added to support cell survival. Brefeldin (10 µg/mL; Sigma-Aldrich, St Louis, Mo) and monensin (2 µmol/L, Sigma-Aldrich) were added for the last 18 hours of culture. SARS-CoV-2 specificity was defined as CD107a<sup>+</sup> IFN-γ<sup>+</sup> cells within CD16<sup>+</sup>CD8<sup>+</sup> T cells or CD16<sup>neg</sup>CD8<sup>+</sup> T cells.

**Transcriptome analysis**

Data from individuals who received mRNA COVID-19 vaccination were obtained from the GSE171964 datasets,<sup>18</sup> which included cellular indexing of transcriptomes and epitopes-sequencing data from 6 different subjects at various time points. The subjects were subdivided into 5 females and 1 male aged 17-26 years old, Hispanic/Latino ethnicity, Native American race. Specifically, single-cell RNA-sequencing (scRNA-seq) results from day 0 and day 28 were extracted and merged using the R package Seurat (R Foundation, Vienna, Austria). CD8<sup>+</sup> cell-based subclustering was performed, followed by batch removal using the Harmony package. The non-naïve CD8<sup>+</sup> cells were then reclustered using the FindClusters function with a resolution of 0.5. Subsequently, the abundance of different cell

populations was determined, and a comparison between day 0 and day 28 was conducted to identify changes influenced by the vaccine. To further analyze the data, a gene set enrichment analysis was performed on Hallmark functions using the escape package in R.

## Statistical analysis

Depending on data, a 2-tailed Student's *t*-test and nonparametric Mann–Whitney test, or ANOVA test were applied to evaluate statistical significance. *P* values <.05 were considered statistically significant (\**P* <.05, \*\**P* <.01, \*\*\**P* <.001). Statistics were calculated using GraphPad Prism 5 software (GraphPad Software, Dotmatics, Boston, Mass).

For detailed methods, please see the Methods section in this article's Online Repository (available at [www.jacionline.org](http://www.jacionline.org)).

## RESULTS

### CD16<sup>+</sup>CD8<sup>+</sup> T cells expand on BNT162b2 COVID-19 vaccination and display an activated phenotype

In the course of analysis of CD8<sup>+</sup> T cells in individuals undergoing BNT162b2 COVID-19 vaccination, we observed a sizable fraction of the CD8<sup>+</sup> T-cell pool expressing CD16. This finding prompted us to study the relative frequencies of CD16<sup>+</sup>CD8<sup>+</sup> T cells in a larger cohort encompassing 30 donors who received both first and second doses of COVID-19 Pfizer-BioNTech vaccine, collecting corresponding pre- and postvaccination blood samples (at baseline: day 0 and 1-week following second dose: day 28). Blood samples were also obtained 6 months following second dose to comparatively analyze the frequency and the activities of this subpopulation of CD8<sup>+</sup> T cells (Fig 1, A). One week after the second vaccine dose, we revealed a marked increase in the frequency of CD16<sup>+</sup> T cells, which was confined to CD8<sup>+</sup> T cells (Fig 1, B). Because it was previously reported that an activation of MAIT cells in response to the BNT162b2 mRNA anti-SARS-CoV-2 vaccine,<sup>11</sup> we stained CD16<sup>+</sup>CD8<sup>+</sup> T cells for the MAIT cell marker Vα7.2 and observed that these cells did not express it (Fig 1, C). The expression level of CD16 on CD8<sup>+</sup> T cells, although lower than on NK cells (Fig 1, D), showed a certain variability between donors. Because in human cytomegalovirus (HCMV)-seropositive individuals, an increase of CD8<sup>+</sup> T cells expressing NK cell–associated receptors<sup>19–21</sup> has been reported, we assessed HCMV serostatus in our cohort by quantification of plasma anti-HCMV IgG and IgM levels. Among 30 individuals enrolled in this study, 18 (60%) were HCMV-seropositive (Fig E2, A, and Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Compared to HCMV<sup>−</sup> vaccinees, HCMV<sup>+</sup> counterpart displayed a larger population of CD16<sup>+</sup>CD8<sup>+</sup> T cells at baseline. However, the increase of CD16-expressing CD8<sup>+</sup> T cells was similar between HCMV seropositive and seronegative groups (Fig E2, B). Similar to NK cells, the majority of CD16<sup>+</sup>CD8<sup>+</sup> T cells expressed NKp80, lacked IL-7 receptor α (CD127), and largely expressed GrzB (Fig 1, E). Accordingly, the expression pattern of the transcription factors T-bet and Eomes revealed that their levels were significantly higher in CD16<sup>+</sup>CD8<sup>+</sup> T cells compared to CD16<sup>neg</sup> counterpart and more similar to NK cells (Fig 1, F). To further support these data, scRNA-seq data from the PBMCs of 6 different individuals who received BNT162b2 mRNA vaccination at 2 different time points (day 0 and day 28) were obtained from the GSE171964

datasets.<sup>18</sup> After merging all data, we focused on 4424 non-naive CD8<sup>+</sup> T cells for further analyses. These cells were then re-clustered, and changes in the abundance of different cell subpopulations between day 0 and day 28 were investigated. Interestingly, we observed, among PBMCs, a single-cell population significantly increased following the vaccination within both CD8<sup>+</sup> and total T lymphocytes (Fig 1, G–I). We then characterized this cell subset by using cell-specific markers in terms of both RNA and surface proteins (Fig E3, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), as CD3<sup>+</sup>CD8<sup>+</sup>CD16<sup>+</sup>CD127<sup>−</sup>CD56<sup>+/−</sup> cells (cluster 5), a profile that is consistent with the one detected by flow cytometry, thus supporting our findings.

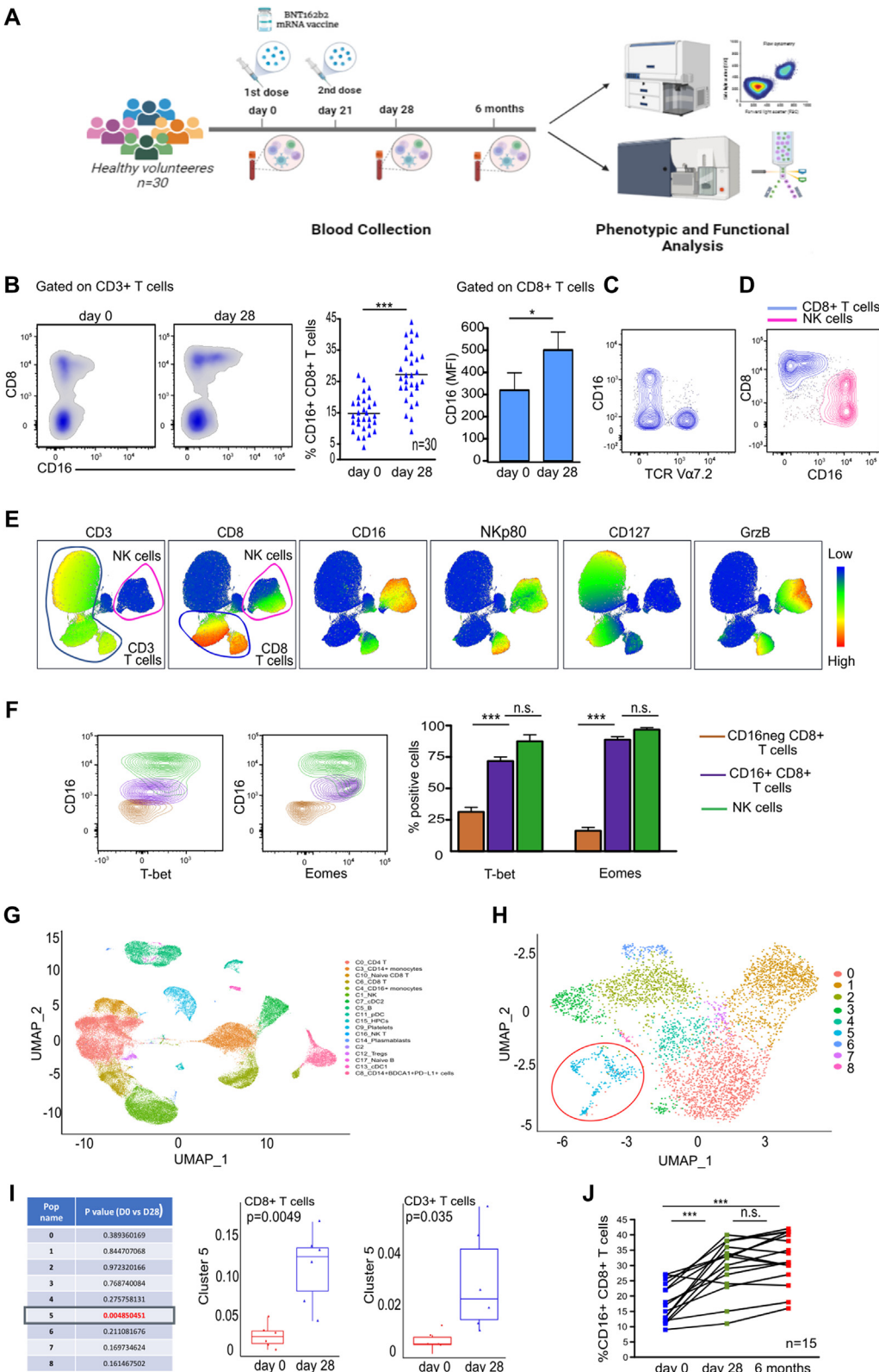
Remarkably, the frequency of these cells remained relatively stable and significantly higher at 6 months following vaccination compared to baseline, indicating that they can persist over time (Fig 1, J).

The percentage of CD16<sup>+</sup>CD8<sup>+</sup> T cells was positively associated with the overall frequency of CD8<sup>+</sup> T-cell compartment (Fig 2, A), and these cells appear in an activated status compared to their CD16<sup>neg</sup> counterparts, as assessed by HLA-DR and CD38 expression (Fig 2, B). Accordingly, when PBMCs from vaccinated donors were analyzed for Helios transcription factors, a marker of T-cell proliferation and activation,<sup>22</sup> the majority of CD16<sup>+</sup>CD8<sup>+</sup> T cells expressed Helios at higher levels than CD16<sup>neg</sup>CD8<sup>+</sup> counterpart (Fig 2, C). Then we performed a pathway and gene set enrichment analysis in the population that we previously identified through scRNA-seq data (as compared to other non-naive CD8<sup>+</sup> T cells), observing an enrichment in specific immune activation hallmark gene sets, thus indicating a functionally relevant correlation with the observed cell phenotype (Fig 2, D). Moreover, CD16<sup>+</sup>CD8<sup>+</sup> T cells were enriched in *IFN1* genes upon vaccination (Fig 2, E), further indicating the pivotal role of type 1 interferon signaling in CD8<sup>+</sup> T-cell response induced by BNT162b2.<sup>23</sup> To strengthen our results, we stimulated *in vitro* donor's PBMCs collected at day 0 with BNT162b2 vaccine and comparatively assessed other conventional vaccine formulations: a protein-based vaccine such as Nuvaxovid containing SARS-CoV-2 antigens (NVX-CoV2373; Novavax, Gaithersburg, Md) and a standard vaccine such as the quadrivalent inactivated influenza vaccine (Vaxigrip Tetra, Sanofi) containing antigens not related to SARS-CoV-2.

In agreement to what was observed in donors undergoing BNT162b2 vaccination, stimulation of PBMCs with this vaccine significantly increased the percentage of CD16<sup>+</sup>CD8<sup>+</sup> T cells in a dose-dependent manner (Fig 2, F and G). These cells expressed NKp80, were highly activated, and possessed a cytotoxic arsenal (Fig 2, F). Remarkably, mRNA, but not other vaccine formulations, increased *in vitro* the proportion of CD16-expressing CD8<sup>+</sup> T cells (Fig 2, H). Taken together, these results demonstrated an increase of activated CD16<sup>+</sup>CD8<sup>+</sup> T cells on BNT162b2 COVID-19 vaccination, prompting us to further characterize this lymphocyte subset in deeper detail with regard to phenotype and function.

### CD8<sup>+</sup> T cells expanded upon BNT162b2 COVID-19 vaccination can acquire ADCC activity

The observation that BNT162b2 COVID-19 vaccination induced the expansion of CD8<sup>+</sup> T cells expressing both CD16 and lytic protein raises the question of whether these cells might



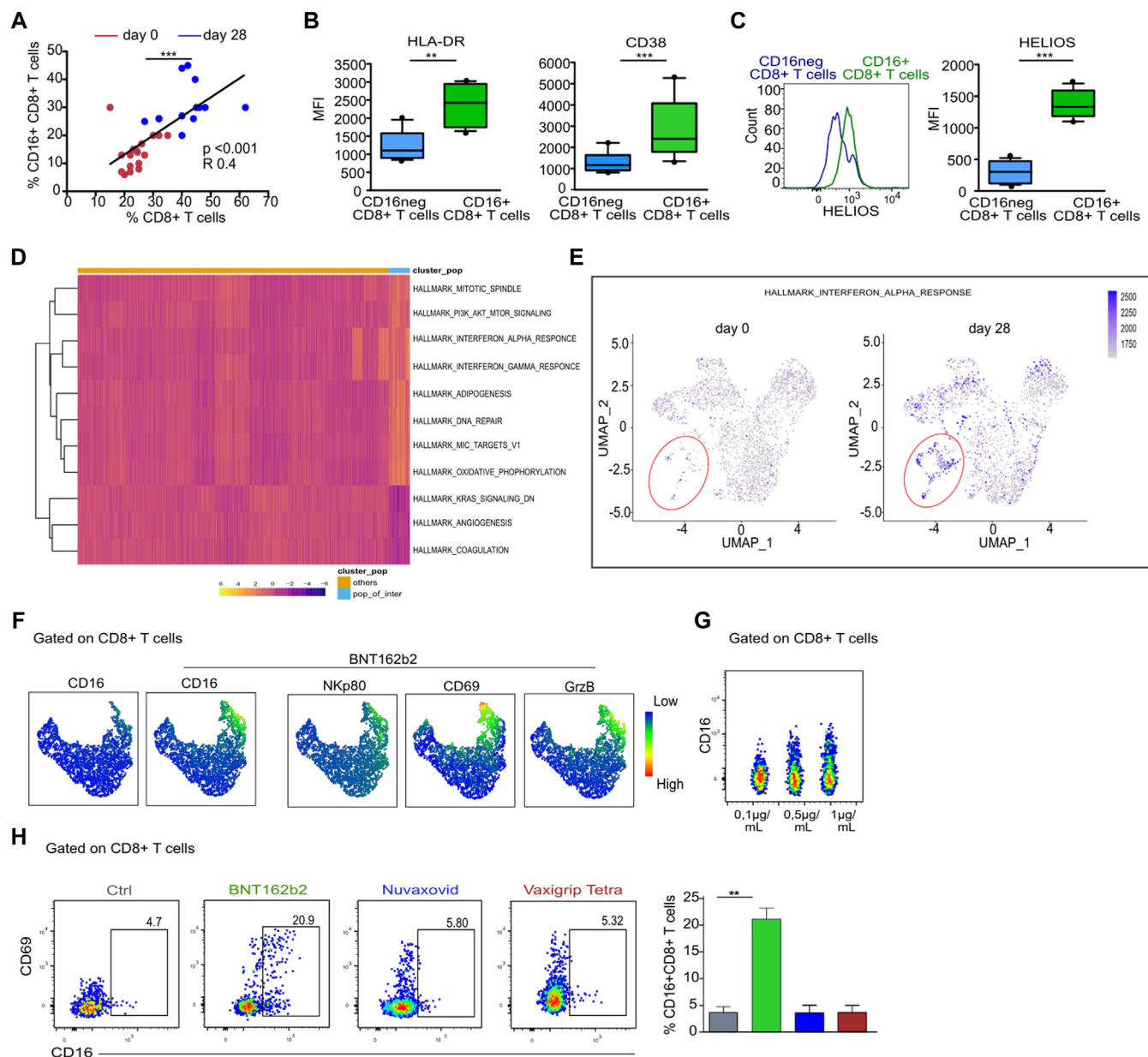
actually mediate ADCC. To test this possibility, populations of interest were sorted from peripheral blood of donors at baseline and at both 1 week and 6 months after receiving the second dose vaccine. The ability to mediate ADCC was evaluated using CTX-coated A549 cell line as target. In addition, specific triggering of CD16 was also performed using P815 cells coated with anti-CD16 mAb. The functional response was also assessed on stimulation with the potent and nonspecific activating agent phorbol 12-myristate 13-acetate and ionomycin (PMA+I) and compared with donor-matched NK cells analyzed in the same conditions. Remarkably, CD16<sup>+</sup>CD8<sup>+</sup> T cells readily acquire and maintain ADCC activity 1 week and 6 months following vaccination, whereas, as expected, no ADCC was detected on CD8<sup>+</sup> T cells at baseline (Fig 3, A, upper panel). Similar results were obtained by CD16-specific triggering in the redirected assay (Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). As expected, donor-matched NK cells showed strong CD107a expression and IFN- $\gamma$  production already at baseline, and this response significantly increased following BNT162b2 COVID-19 vaccination (Figs 3, A, lower panel, and E4). These results demonstrated that stimulation through CD16 can elicit a functional response on CD16<sup>+</sup>CD8<sup>+</sup> T cells expanded following BNT162b2 COVID-19 vaccination, and, interestingly, this response can be retained up to 6 months following vaccination. ADCC mediated by NK cells was also enhanced upon vaccination, and this observation supports and is in line with the recent evidence that BNT162b2 vaccination induces highly functional antibodies mediating strong ADCC activity by NK cells.<sup>24</sup> However, no correlation was observed between frequency of CD16<sup>+</sup>CD8<sup>+</sup> T cells and anti-Spyke IgG titer (Fig E5, A and B, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although the frequency of responding cells on total CD8<sup>+</sup> T cells was lower compared to NK cells, after adjusting for the frequency of CD16 expression in these populations, CD16-mediated CD8<sup>+</sup> T-cell activity was similar to that of CD16<sup>+</sup> NK cells (Fig 3, B). In agreement with these results, gene enrichment analyses related to IFN- $\gamma$  signaling revealed the activation of interferon-activated genetic programs in CD16<sup>+</sup>CD8<sup>+</sup> effector T cells expanded on vaccination (Fig 3, C).

Moreover, when CD8<sup>+</sup> T cells were dissected according to the expression of NKp80, highly expressed by CD16<sup>+</sup>CD8<sup>+</sup> T cells, both degranulation and cytokine production were mainly confined to NKp80<sup>+</sup> fraction (Fig 3, D). A stronger response was also observed in CD16<sup>+</sup>CD8<sup>+</sup> T cells on PMA+I stimulation, suggesting that, in general, this subset of CD8<sup>+</sup> T cells is more reactive and might undergo a stronger activation compared to conventional CD8<sup>+</sup> T cells (Fig E6 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

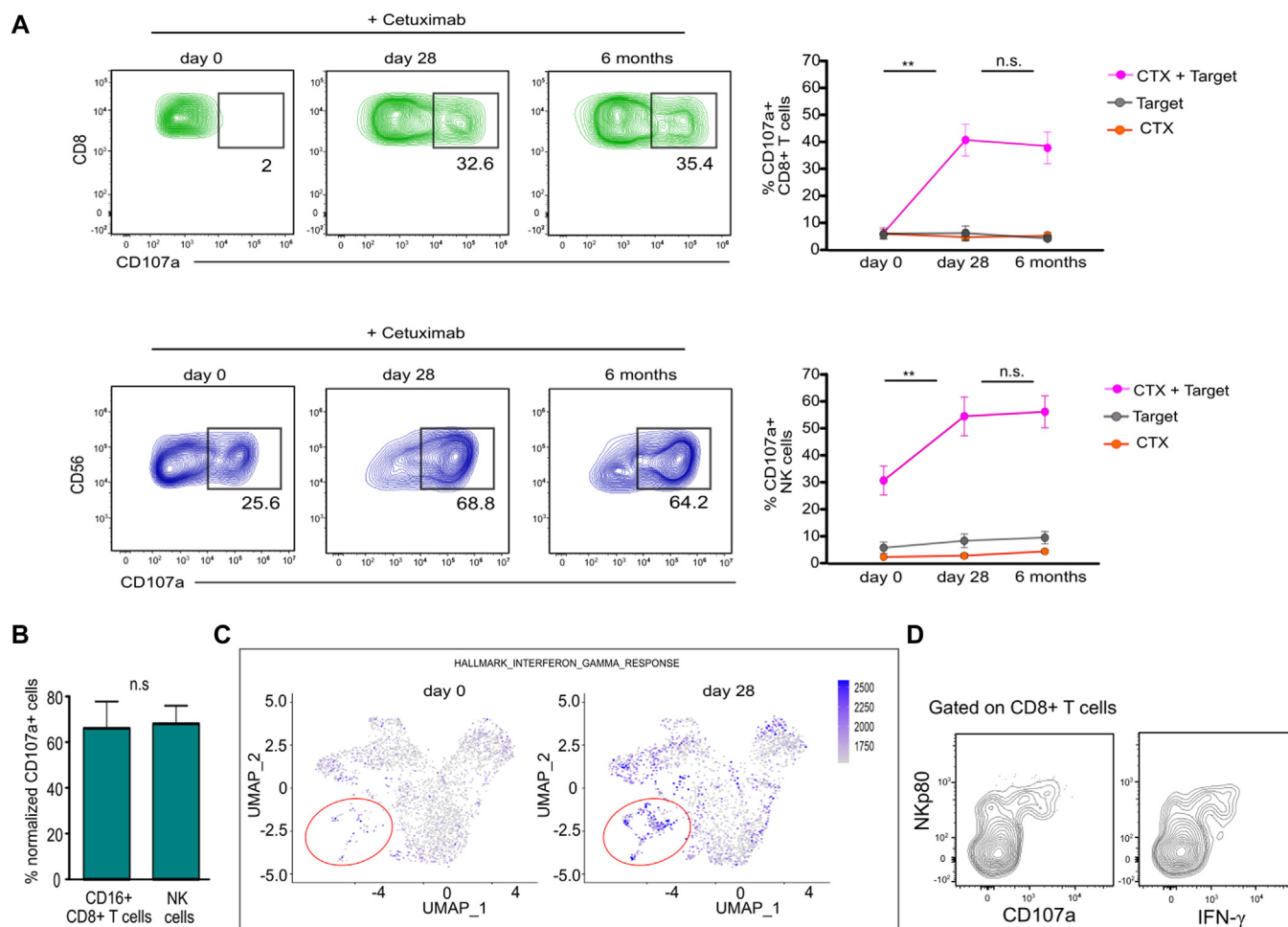
### CD16<sup>+</sup>CD8<sup>+</sup> T cells are terminally differentiated effector cells

Because of the significant expansion and activation of CD16<sup>+</sup>CD8<sup>+</sup> T cells upon vaccination, we next investigated the detailed phenotype of these cells analyzing a panel of markers involved in T-cell functions. For these analyses, cells from vaccinated donors were sorted into 3 populations: (1) CD8<sup>+</sup> T cells lacking expression of CD16; (2) CD8<sup>+</sup> T cells expressing CD16; (3) CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. To assess the differentiation status of CD16<sup>+</sup>CD8<sup>+</sup> T-cell subset, we stained for CD45RA, CCR7, CD27, and CD28. Expression of CD45RA in the absence of CCR7, CD27, and CD28 was the dominant pattern among the CD16<sup>+</sup>CD8<sup>+</sup> T cells (Fig 4, A), consistent with effector memory re-expressing CD45RA (EMRA) phenotype and thus a terminally differentiated status. In agreement with their late-stage differentiated effector phenotype, the majority of CD16<sup>+</sup>CD8<sup>+</sup> T cells expressed the senescence marker CD57, which was significantly higher compared to CD16<sup>neg</sup>CD8<sup>+</sup> T cells (Fig 4, A). Also, compared to CD16<sup>neg</sup>CD8<sup>+</sup> T cells, this population of CD8<sup>+</sup> T cells expressed a higher level of CD161, promyelocytic leukemia zinc finger transcription factor (PLZF), and CD49d, thus highlighting an innate-like phenotype distinguished from conventional CD8<sup>+</sup> T cells (Fig 4, B). In this regard, it is noteworthy that compared to other CD8<sup>+</sup> T cells, CD16<sup>+</sup>CD8<sup>+</sup> T cells express CD8 $\alpha$  at much lower level than CD8 $\beta$  chain, becoming more akin to unconventional T cells and NK cells (Fig 4, B). To investigate the relative levels of clonality among CD16<sup>+</sup> and CD16<sup>neg</sup>CD8<sup>+</sup> T-cell subsets, we evaluated the T-cell receptor (TCR) V $\beta$  distribution in these populations by using an array of 24 antibodies specific for different TCR V $\beta$ . In the majority of donors analyzed, CD16<sup>+</sup>CD8<sup>+</sup> T-cell subset displays a restricted TCR V $\beta$  repertoire dominated by a single (or a few) TCR V $\beta$  (Fig 4, C). Conversely, CD16<sup>neg</sup>CD8<sup>+</sup> T cells showed a broader TCR V $\beta$  repertoires (Fig 4, C). Taken together, these data indicate that CD16<sup>+</sup>CD8<sup>+</sup> T cells represent a population of terminally differentiated effector T cells that phenotypically appear as antigen-experienced lymphocytes. Then, we assessed whether CD16<sup>+</sup>CD8<sup>+</sup> T cells displayed specificity for SARS-CoV-2. To this aim, PBMCs from donors at day 0, day 28, and 6 months were stimulated with a pool of SARS-CoV-2 peptides and the proportion of SARS-CoV-2-reactive CD16<sup>+</sup>CD8<sup>+</sup> T cells (as percentage of CD107<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells) was determined after 24 hours. On average 5-7% of the CD16<sup>+</sup>CD8<sup>+</sup> T cells released both CD107a and IFN- $\gamma$  1 week after the second dose (day 28) (Fig 4, D). These cells remained detectable after 6 months, showing a significantly higher frequency compared to baseline (Fig 4, D). Moreover, when comparing the frequencies of

CD8<sup>+</sup> T cells (blue) analyzed at day 28 (n = 15). (E) A t-distributed stochastic neighbor embedding (t-SNE) analysis of lymphocytes to assess the expression levels of CD16, NKp80, CD127, and GrzB on CD8<sup>+</sup> T cells and NK cells, assessed on 10 donors at day 28. (F) Expression of T-bet and Eomes assessed on CD16<sup>+</sup>CD8<sup>+</sup> T cells, CD16<sup>neg</sup>CD8<sup>+</sup> T cells and NK cells. Bars represent mean percentage  $\pm$  SEM of positive cells (n = 6); \*\*\*P < .001. (G-I) Uniform manifold approximation and projection (UMAP) visualizations of 4424 single cells obtained from scRNA-seq of 6 different individuals who received mRNA COVID vaccination at 2 different time points (day 0 and day 28) and profiled with cellular indexing of transcriptomes and epitopes-sequencing. Non parametric Mann-Whitney test was performed in I. Box plot indicates the median and interquartile range. Whisker bars the 10th and 90th percentiles. \*\*P < .01, \*\*\*P < .05. Bars represent frequency  $\pm$  SEM of cluster 5 population between day 0 and day 28 among CD8<sup>+</sup> T cells and CD3<sup>+</sup> T cells. (J) CD16<sup>+</sup>CD8<sup>+</sup> T-cell frequency analyzed in 15 donors at day 0, day 28, and 6 months following vaccination; \*\*\*P < .001. MFI, mean fluorescent intensity; ns, not significant.



**FIG 2.** Immunophenotyping and transcriptome data show the activation status of CD16<sup>+</sup>CD8<sup>+</sup> T cells. **(A)** Correlation between the frequency of CD16<sup>+</sup>CD8<sup>+</sup> T-cell subset and CD8<sup>+</sup> T cells within CD3<sup>+</sup> T-cell population at day 0 and day 28 (n = 27); \*\*\*P < .001. **(B)** Expression of HLA-DR and CD38, as MFI, on CD16<sup>+</sup>CD8<sup>+</sup> T cells and CD16<sup>neg</sup>CD8<sup>+</sup> T cells from 5 independent experiments. Box plot indicates the median and interquartile range and whisker bars the 10th and 90th percentiles. Nonparametric Mann-Whitney test was performed (n = 10); \*\*P < .01, \*\*\*P < .05. **(C)** Expression of Helios on CD16<sup>+</sup>CD8<sup>+</sup> T cells and CD16<sup>neg</sup>CD8<sup>+</sup> T cells assessed by flow cytometry. Box plot indicates the median and interquartile range and whisker bars the 10th and 90th percentile. Nonparametric Mann-Whitney test was performed (n = 10); \*\*\*P < .001. **(D)** Pathway and gene set enrichment analysis on the population identified through scRNA-seq data (Fig 1, G-I) of specific immune activation hallmark gene sets. **(E)** Gene enrichment analysis of the activation of IFN- $\alpha$ -activated genetic programs in the CD16<sup>+</sup>CD8<sup>+</sup> T-cell subset at day 0 and day 28 following vaccination. **(F)** Expression of CD16, NKp80, CD69, and GrzB was evaluated by a t-SNE analysis on CD8<sup>+</sup> T cells following stimulation for 7 days with 1  $\mu$ g/mL of BNT162b2 COVID-19 vaccine (n = 7). **(G)** Dose-dependent expression of CD16 on CD8<sup>+</sup> T cells stimulated with 1  $\mu$ g/mL, 100 ng/mL, or 500 ng/mL of BNT162b2 COVID-19 vaccine for 7 days (n = 7). **(H)** Representative dot plots and relative statistical analysis showing the expression of CD16 on CD8<sup>+</sup> T cells following stimulation with different vaccine formulations: BNT162b2, Nuvaxovid, or influenza vaccine for 7 days (n = 7); \*\*P < .01.



**FIG 3.** CD16<sup>+</sup>CD8<sup>+</sup> T cells exert ADCC and are enriched with IFN- $\gamma$  genes. **(A)** Representative dot plots showing the expression of CD107a following CTX-mediated cytotoxicity by CD8<sup>+</sup> T cells (upper) and NK cells (lower) collected at day 0, day 28, and 6 months following vaccination. Graphs represent mean  $\pm$  SEM of CD107a<sup>+</sup> cells ( $n = 5$ );  $**P < .01$ . Cells cultured with target or with CTX were used as controls. **(B)** Bars represent normalized frequency  $\pm$  SEM of CD16<sup>+</sup>CD8<sup>+</sup> T cells and NK cells responding with CD107a to CD16 triggering ( $n = 10$ ). **(C)** Gene enrichment analyses of the activation of IFN- $\gamma$ -activated genetic programs in the CD8<sup>+</sup> T-cell subset at day 0 and day 28 following vaccination. **(D)** CD107a and IFN- $\gamma$  expression assessed on CD8<sup>+</sup> T cells collected at day 28 based on NKp80 expression on CD16 stimulation.

SARS-CoV-2-reactive T cells between CD16<sup>+</sup> and CD16<sup>neg</sup>CD8<sup>+</sup> T-cell compartments, we detected a significant enrichment in CD16<sup>+</sup>CD8<sup>+</sup> T cells.

### CD16<sup>+</sup>CD8<sup>+</sup> T cells express functional NK-cell receptors

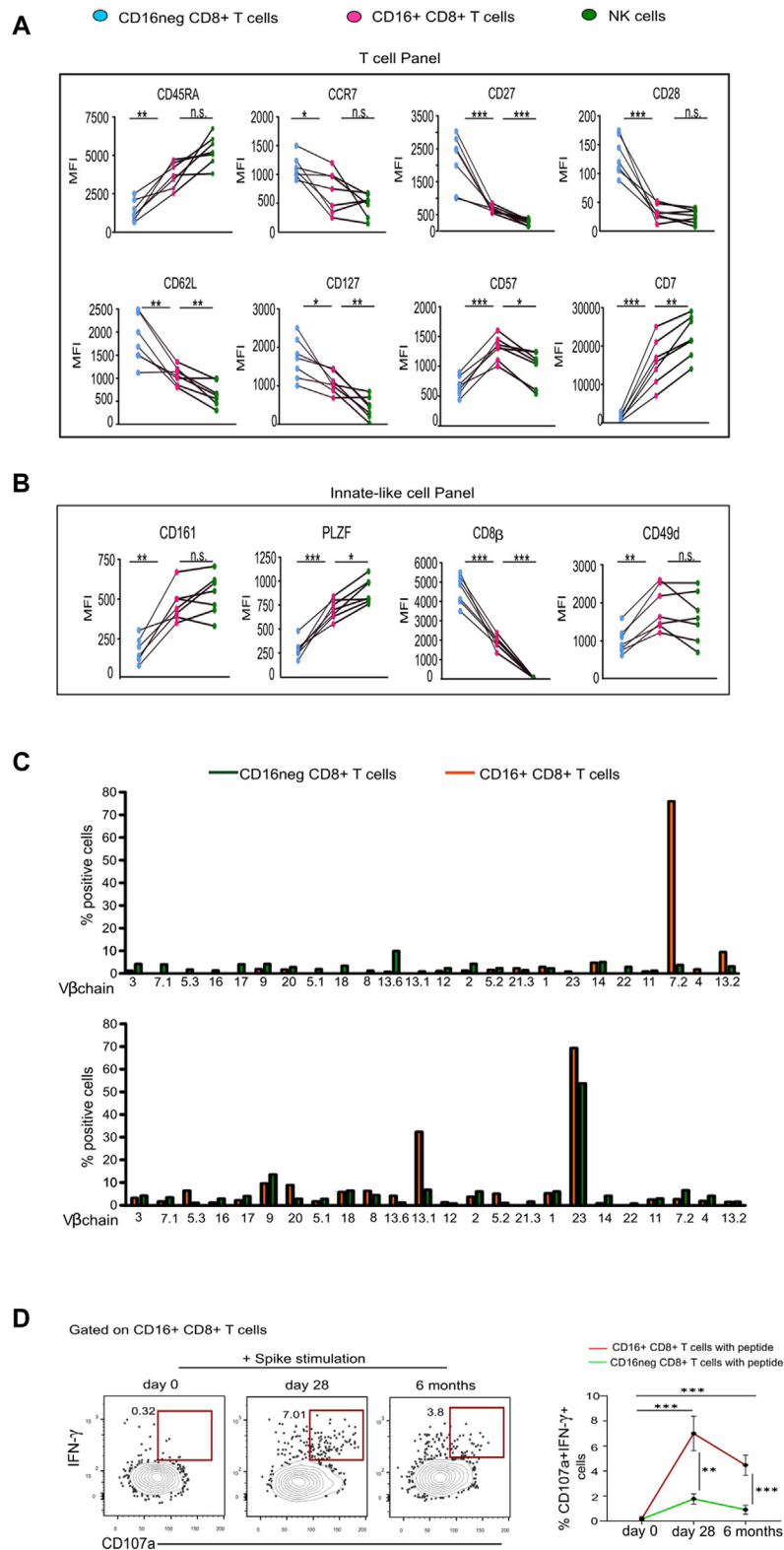
In addition to CD16 and NKp80, we then determine the expression profile of major activating and inhibitory NK-cell receptors. All CD16<sup>+</sup>CD8<sup>+</sup> T cells displayed DNAX accessory molecule-1 (DNAM-1) and NK group 2, member D (NKG2D) surface expression (Fig 5, A), which were significantly more expressed compared to CD16<sup>neg</sup>CD8<sup>+</sup> T cells. CD16<sup>+</sup>CD8<sup>+</sup> T cells were also positive for inhibitory killer cell Ig-like receptors (KIRs) and CD94/ NK group 2, member A (NKG2A) inhibitory receptor (Fig 5, A). Natural cytotoxicity receptor (NCR) expression is thought to be mainly restricted to NK cells and innate lymphoid cells.<sup>25</sup> Nevertheless, we could observe that CD16<sup>+</sup>CD8<sup>+</sup> T cells express a significant level of NKp30 that was undetectable on CD16<sup>neg</sup> counterpart (Fig 5, A). Thus, we

carried out functional experiments to investigate the response evoked upon specific NK activating receptor triggering. The responses were compared with the activity of donor-matched CD16<sup>neg</sup>CD8<sup>+</sup> T cells similarly stimulated. CD16<sup>+</sup>CD8<sup>+</sup> T cells largely responded with degranulation after NK receptors triggering (Fig 5, B), clearly indicating that this subset of CD8<sup>+</sup> T lymphocytes display NK-like functionality.

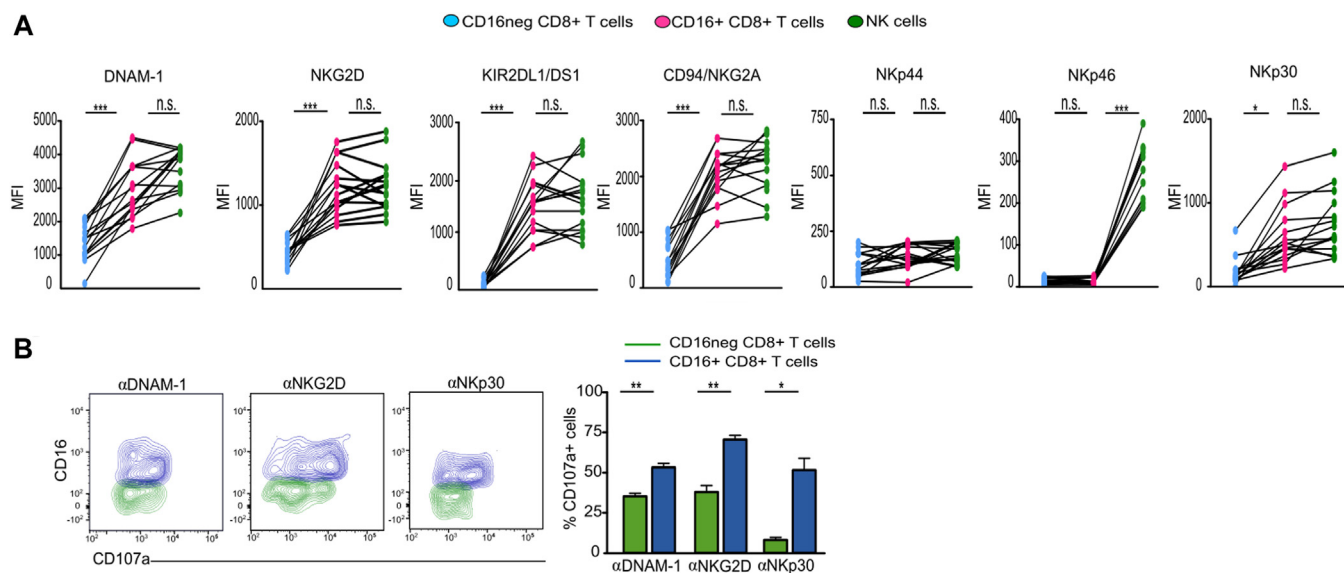
### NKp80<sup>+</sup>CD8<sup>+</sup> T cells can upregulate CD16 and NKp30 upon IL-15 stimulation

CD16<sup>+</sup>CD8<sup>+</sup> T cells show an EMRA phenotype and express high levels of the NK-cell marker NKp80 (Figs 1, E, and 4, A). NKp80 defines a reactive subset of CD8<sup>+</sup> T cells showing an EMRA phenotype and nearly absent from naive T and central memory T cells (Fig 6, A).<sup>26</sup>

Starting from these observations, we investigated whether NKp80<sup>+</sup>CD8<sup>+</sup> T cells might upregulate CD16 surface expression when cultured in the presence of IL-15, a key cytokine for the development of NK cells. To address this issue, CD8<sup>+</sup> T lymphocytes



**FIG 4.** CD16<sup>+</sup>CD8<sup>+</sup> T cells are late-stage effector T cells with a restricted TCR V $\beta$  repertoire. Scatter plots showing the expression, indicated as MFI, of the indicated markers associated with T-cell profile (**A**) and innateness (**B**) on CD16<sup>+</sup>CD8<sup>+</sup> T cells, CD16<sup>neg</sup>CD8<sup>+</sup> T cells and NK cells ( $n = 15$ ); \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ . (**C**) TCR V $\beta$  landscape assessed by flow cytometry showed the diversity of TCR  $\beta$  chain on CD16<sup>+</sup>CD8<sup>+</sup> T cells and CD16<sup>neg</sup>CD8<sup>+</sup> T cells from 4 representative volunteers collected at day 28 following vaccination. (**D**) Representative dot plots and relative statistical analysis of SARS-CoV-2 antigen specificity measured at day 0, day 28, and 6 months following vaccination on CD16<sup>+</sup>CD8<sup>+</sup> and CD16<sup>neg</sup>CD8<sup>+</sup> T cells stimulated for 24 hours with a peptide pool encompassing the complete S protein and showed as percentage of CD107a<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells; \*\* $P < .01$ , \*\*\* $P < .001$ .



**FIG 5.** CD16<sup>+</sup>CD8<sup>+</sup> T cells display functional NK-cell receptors. **(A)** Scatter plots showing the expression of NK cell-associated activating and inhibitory receptors on CD16<sup>+</sup>CD8<sup>+</sup> T-cell and CD16<sup>neg</sup>CD8<sup>+</sup> T-cell populations collected at day 28 (n = 15); \*P < .05, \*\*\*P < .001. **(B)** Representative dot plots and relative statistical analysis showing the expression of CD107a on CD16<sup>+</sup>CD8<sup>+</sup> T cells and CD16<sup>neg</sup>CD8<sup>+</sup> T cells following specific triggering of activating receptors DNAM-1, NKG2D, and NKp30. Bars represent frequency ± SEM of CD107a<sup>+</sup> cells (n = 5); \*P < .05; \*\*P < .01.

were stringently sorted using fluorescence-activated cell sorting from peripheral blood as NKp80<sup>+</sup>CD8<sup>+</sup> and NKp80<sup>neg</sup>CD8<sup>+</sup> T cells and cultured *in vitro* with recombinant IL-15 (Fig 6, B). We observed that sorted NKp80<sup>+</sup>CD8<sup>+</sup> T cells showed certain evidence of CD16 surface expression at baseline, which significantly increased at day 6 (Fig 6, B), while NKp80<sup>neg</sup>CD8<sup>+</sup> T cells did not upregulate CD16 in the same culture condition (Fig 6, B). IL-15 has been reported to be able upregulate the expression of different NK receptors on CD8<sup>+</sup> T cells.<sup>27,28</sup> Thus besides CD16, we asked whether NKp80<sup>+</sup>CD8<sup>+</sup> T cells might upregulate also NKp30 on stimulation. As shown in Fig 6, B, upregulation of CD16 surface expression observed in sorted NKp80<sup>+</sup>CD8<sup>+</sup> T cells was accompanied by an increase of NKp30 expression level while NKp80<sup>neg</sup> counterpart did not show this classical NK-cell marker. The functional relevance of NKp80 has been previously assessed on CD8<sup>+</sup> T cells.<sup>26</sup> Here, given the coexpression of NKp80 and CD16, we stimulated freshly purified CD8<sup>+</sup> T cells with anti-NKp80 mAb together with anti-CD16 mAb and monitored intracellular expression of IFN-γ and the appearance of the degranulation marker CD107a on the cell surface. Stimulation by anti-CD16/anti-NKp80 mAbs resulted in a marked increase of both degranulation and IFN-γ production when compared to anti-CD16 alone (Fig 6, C). Thus, engagement of NKp80 in combination with CD16 resulted in an additional effect, potentially leading to a synergistic increase of the responses of CD8<sup>+</sup> T-cell subset expanded on vaccination.

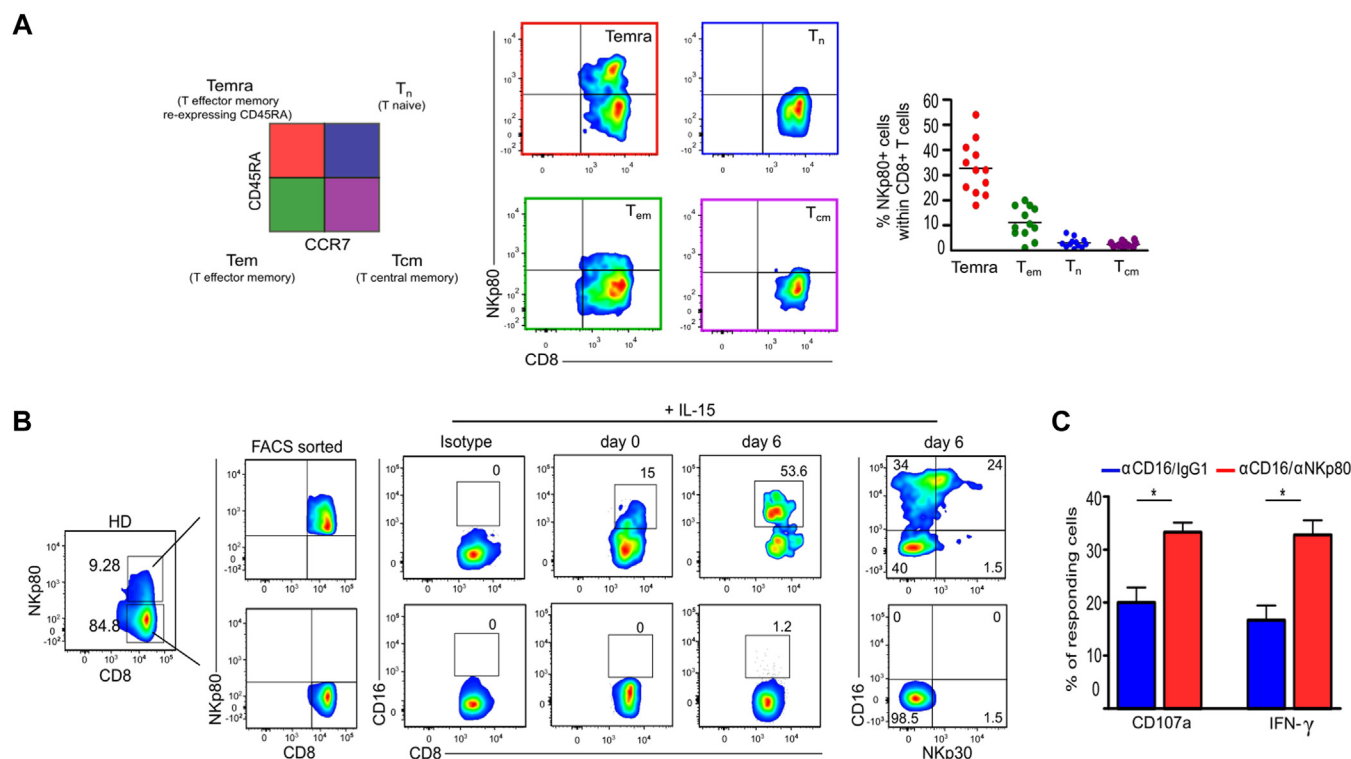
### CD16<sup>+</sup>CD8<sup>+</sup> T cells express the inhibitory receptor GPR56 that limits their effector functions

G protein-coupled receptor 56 (GPR56) has been identified as an inhibitory receptor expressed on human NK cells.<sup>29</sup> However, its expression has also been reported on a subset of cytotoxic

T lymphocytes showing a terminally differentiated EMRA phenotype.<sup>30</sup> In agreement with the uniform presence of GPR56 on mature circulating NK cells expressing CD16, all CD16<sup>+</sup>CD8<sup>+</sup> T cells also express GPR56, which is conversely not present in CD16<sup>neg</sup> counterpart (Fig 7, A). Notably, activation of primary NK cells downregulates the expression of GPR56.<sup>29,31</sup> Accordingly, stimulation of CD16<sup>+</sup>CD8<sup>+</sup> T cells for 30 minutes, 1 hour, and 3 hours with the indicated amounts of PMA resulted in a progressive loss of GPR56 surface expression and, concomitantly, in the downregulation of CD16 (Fig 7, B). Activation of NK-like CD8<sup>+</sup> T cells might also occur through cytokines. We therefore incubated PBMCs for 12 hours with IL-2 or IL-15. IL-2 treatment induced a progressive reduction of GPR56 expression, and an even stronger effect was detected by culturing cells with IL-15 (Fig 7, C). GPR56 forms complexes with the tetraspanin proteins CD81 at the NK-cell surface that negatively regulate their effector functions.<sup>29</sup> Because CD16<sup>+</sup>CD8<sup>+</sup> T cells coexpress GPR56 and CD81 (Fig 7, D), we investigated whether GPR56-CD81 complex could affect CD16-mediated CD8<sup>+</sup> T cells' response by stimulating with anti-GPR56 mAb, which is able to cause a rapid dissociation of the GPR56-CD81 complex.<sup>29</sup> Similar to NK cells, CD16-mediated CD8<sup>+</sup> T-cell cytolytic activity was significantly enhanced in the presence of anti-GPR56 mAb (Fig 7, E). These data indicate that GPR56 is strongly expressed by CD16<sup>+</sup>CD8<sup>+</sup> T cells and might regulate ADCC by these highly reactive cytotoxic effector lymphocytes.

### DISCUSSION

Multiple studies have analyzed immune responses to BNT162b2 vaccine and demonstrated significant cell-mediated and humoral immune responses to the vaccine.<sup>23</sup> Neutralizing antibodies can block virus binding to angiotensin converting



**FIG 6.** NKp80<sup>+</sup>CD8<sup>+</sup> T cells acquire the expression of functional CD16 and NKp30 in the presence of IL-15. **(A)** Analysis of the distribution of NKp80<sup>+</sup> cells among CD8<sup>+</sup> T-cell subpopulations dissected according to CD45RA and CCR7 expression. **(B)** Using fluorescence-activated cell sorting (FACS), NKp80<sup>+</sup> and NKp80<sup>neg</sup>CD8<sup>+</sup> T cells were sorted from PBMCs at day 0 and expression of CD16 and NKp30 was analyzed following 6 days of culture with IL-15. **(C)** Bars represent frequency  $\pm$  SEM of CD107a<sup>+</sup> and IFN- $\gamma$ <sup>+</sup> cells assessed on CD8<sup>+</sup> T cells and NK cells following cross-linking of CD16 alone and in combination with NKp80 (n = 5); \*P < .05.

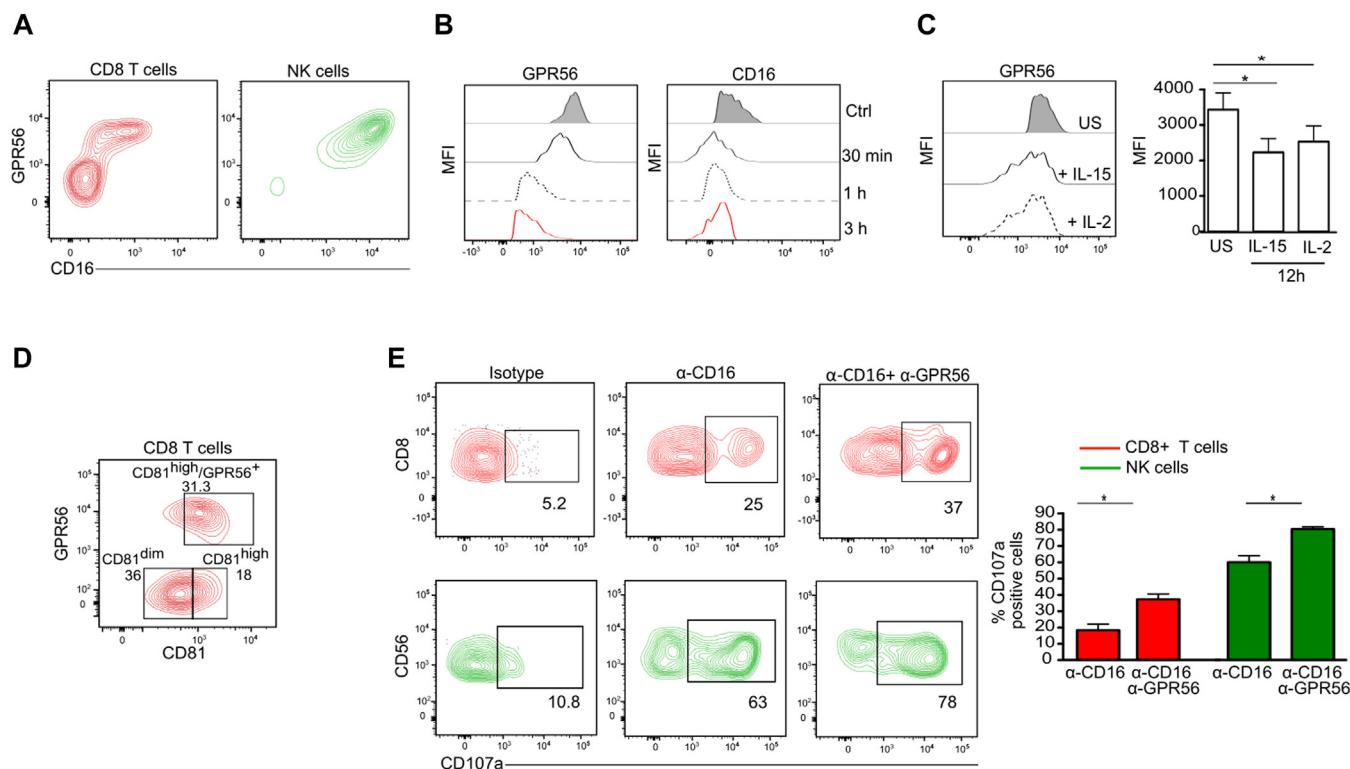
enzyme-2 (ACE-2), thus preventing virus entry and reducing SARS-CoV-2 transmission.<sup>32</sup> However, in animal models, it has been demonstrated that ADCC is capable of preventing disease even without neutralizing activity of antibodies.<sup>33-35</sup>

In the current study, we reported a significant expansion of unconventional CD8<sup>+</sup> T-cell subset bearing CD16 in the peripheral blood of individuals undergoing BNT162b2 vaccination.

This CD8<sup>+</sup> T-cell subset shows an activated phenotype and acquires strong ADCC activity via CD16, thus acting independently of the TCR. On the other hand, approximately 5-7% of the activated CD16<sup>+</sup>CD8<sup>+</sup> T cells respond to stimulation with SARS-CoV-2 peptides, indicating a role of TCR activation in their generation. A study, investigating the innate and adaptive responses to BNT162b2 in mice, reported that NK cells and CD8<sup>+</sup> T cells in the draining lymph nodes are the major producers of circulating IFN- $\gamma$ , a cytokine critically involved in the protective response against SARS-CoV-2 infection, and that T-cell response is dependent on signaling via IFN $\alpha$ .<sup>23</sup> IFN- $\alpha$  is critical for the generation of effector memory cells in response to viral infection<sup>36</sup> and contributes to the innate function of CD8<sup>+</sup> T cells, directly<sup>37</sup> or indirectly via the upregulation of specific cytokines, such as IL-15, on antigen-presenting cells.<sup>38,39</sup> Here, we found that CD16<sup>+</sup>CD8<sup>+</sup> effector T cells, which increase following vaccination, exhibit innate features and are significantly enriched in both IFN- $\alpha$  and IFN- $\gamma$  genetic programs compared to other CD8<sup>+</sup> T lymphocytes. In addition, they express high level of

NKp80, a typical NK-cell marker and can differentiate from NKp80<sup>+</sup>CD8<sup>+</sup> T cells on stimulation with IL-15. The differentiating process is supported by the notion that, unlike NKp80<sup>neg</sup> subpopulation, NKp80<sup>+</sup>CD8<sup>+</sup> T lymphocytes, as T<sub>EMRA</sub> cells, can undergo very limited proliferation, if any.<sup>28,40</sup>

Along with NKp80, this CD8<sup>+</sup> T-cell subset, expanded upon vaccination, displays other innate activating receptors, including NKp30, whose specific triggering evokes a vigorous response confirming the effective cytotoxic potential of these cells. Because of these innate receptors and of their strong ADCC capability, this abundant subset of CD16<sup>+</sup>CD8<sup>+</sup> can potentially exert, as NK cells, a protective activity also against many other viral diseases. In addition, innate T cells, expressing receptors that are commonly found on NK cells, are gaining increasing interest also for cancer immunosurveillance. Growing evidence indicates that these cells accumulate in tumors and can eliminate transformed cells with a low mutational burden because they are able to recognize unmutated antigens exposed on their surface.<sup>41</sup> In addition, this CD8<sup>+</sup> T-cell subpopulation, combining the characteristics of both CD8<sup>+</sup> T and NK cells, can kill target cells by exploiting their dual-recognition capacity.<sup>27</sup> Thus, the capability of mRNA-based vaccines to induce CD8<sup>+</sup> T cells with NK-like features that persist over time could have important implications in counteracting not only infectious disease but, possibly, also the initial phases of cancer development. Importantly, these cells can be engineered both *ex vivo* and *in vivo* to enhance their activity via



**FIG 7.** Cross-linking of GPR56 enhances CD16-mediated activation of CD8<sup>+</sup> T cells. **(A)** Expression of GPR56 on CD8<sup>+</sup> T cells and NK cells assessed by flow cytometry according to CD16 expression. **(B)** Expression of CD16 and GPR56 on CD8<sup>+</sup> T cells following stimulation with PMA+I at the indicated time points. **(C)** Expression of GPR56 on CD8<sup>+</sup> T cells stimulated for 12 hours with IL-15 or IL-2. Bars represent MFI of GPR56<sup>+</sup> cells (n = 4). \*P < .05. **(D)** Expression level of CD81 on GPR56<sup>+</sup>/neg CD8<sup>+</sup> T cells assessed by flow cytometry. **(E)** Representative dot plots and relative statistical analysis showing the expression of CD107a by CD16<sup>+</sup> CD8<sup>+</sup> T cells (red) or NK cells (green) following cross-linking of CD16 alone and in combination with GPR56. Bars represent mean percentage ± SEM of CD107a<sup>+</sup> cells (n = 5); \*P < .05.

engagement of the IL-15.<sup>42</sup> The identification of a specific subset of CD8<sup>+</sup> T cells expressing Nkp80 that, upon stimulation by IL-15, is capable of generating T cells with innate features could be particularly useful to improve therapeutic strategies involving these cells. Nkp80 is an activating receptor in CD8<sup>+</sup> T cells and its ligation, besides augmenting CD3-stimulated degranulation,<sup>28</sup> increases CD16-mediated activation, potentially leading to a synergistic increase in the responses of vaccine-expanded CD8<sup>+</sup> T cells. However, these potent cytotoxic mechanisms need to be tightly controlled. In the present work, we also observed that CD16<sup>+</sup>CD8<sup>+</sup> T cells express the inhibitory complex GPR56/CD81, commonly found in human NK cells. An interesting study, analyzing the impact of mild COVID-19 on the immune response to influenza vaccination, reveals that GPR56<sup>+</sup> T cells induced by the previous SARS-CoV-2 infection, could be a source of elevated IFN-γ after influenza vaccination. This more potent interferon response is sustained by IL-15 produced by monocytes early after vaccination.<sup>43</sup> It would be interesting to investigate whether GPR56<sup>+</sup>CD16<sup>+</sup>CD8<sup>+</sup> T cells, expanded on BNT162b2 vaccination, could imprint the immune status of an individual, with the potential to impact responses to future perturbations.

In summary, our study provides new insights into the effects of BNT162b2 vaccine in humans. In our view, the self-adjuvant properties of mRNA vaccine, capable of stimulating the secretion

of large amounts of type I IFN and proinflammatory cytokines, synergize with a strong HLA class I-restricted TCR stimulation by mRNA vaccine antigens that, differently from other vaccine formulations, are largely processed by the endogenous pathway: the combination of these 2 factors, peculiar of mRNA vaccines, is likely to result in the induction of CD8<sup>+</sup> T cells endowed with innate functions. This novel information could provide a more accurate rationale for the use of mRNA-based vaccines against different diseases.

## DISCLOSURE STATEMENT

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### Key messages

- BNT162b2 vaccination induces the expansion of CD8<sup>+</sup> T cells expressing CD16, a low-affinity FcR for IgG.
- CD16<sup>+</sup>CD8<sup>+</sup> T cells display potent ADCC and innate receptor-associated cytotoxicity.
- NKp80<sup>+</sup>CD8<sup>+</sup> T cells generate CD16<sup>+</sup>CD8<sup>+</sup> NK-like T cells following stimulation with IL-15.

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