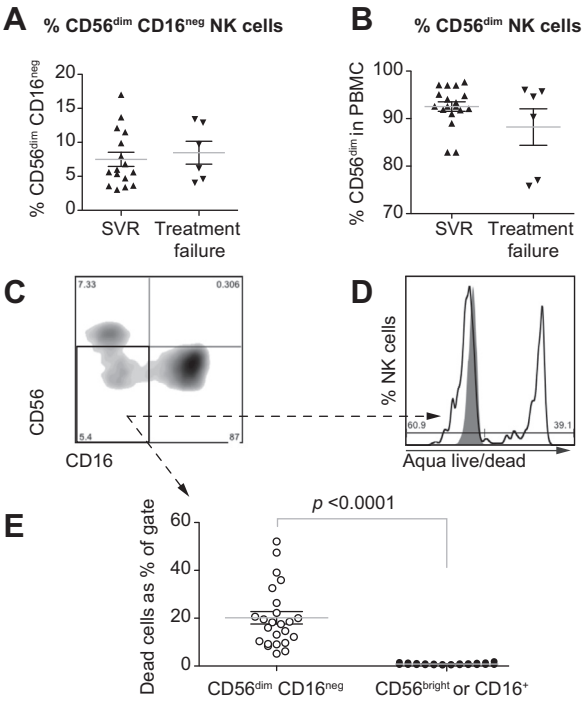

Fig. 1. $CD56^{dim}CD16^{neg}$ NK cells, HCV treatment outcome, and the impact of live/dead staining. (A) Pretreatment $CD56^{dim}CD16^{neg}$ NK cells in 24 HCV patients who have achieved SVR (triangles) and failed treatment (inverted triangles, mean 7.4 and 8.4%, respectively p = not significant). (B) $CD56^{dim}$ NK cell proportion and treatment outcome (mean 92.5 and 88.4%, respectively p = not significant). (C) CD56 and CD16 expression in an NK cell population from which dead cells have not been excluded. (D) Alive/dead staining on $CD56^{dim}CD16^{neg}$ NK cells highlighted quadrant in (C) shown by black line. Solid grey population represents the cells from the other 3 quadrants of $CD56^{bright}$ or $CD16^{+}$ NK cells. (E) The proportion of NK cells which are dead, stain positive in $CD56^{dim}CD16^{neg}$ gate and from the remaining $CD56^{bright}$ or $CD16^{+}$ gates. Mean and SEM shown.

$CD19^{+}$ cells should be excluded to prevent monocyte and B cell contamination. Description and representative FACS plots of fidelity minus, isotype controls and mean fluorescent intensity with mean cell expression values should be included. We believe that this will ensure a robust body of literature with flow data that is reasonably comparable between studies, strengthening our understanding of this important cell type in chronic HCV infection without increasing unnecessary conflicts within the literature.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Reply to: "Flow cytometry makes all the difference"

Does it? No, it doesn't

To the Editor:

We thank Pembroke and colleagues for the comments to our paper which, we are pleased to note, is generating interest in the small community of liver immunologists. Contrary to our data, they found no difference in the percentage of $CD56^{dim}/CD16^{neg}$ NK cells between patients developing a sustained virological response (SVR) and those who failed treatment, and hypothesize that the higher proportion of $CD56^{dim}/CD16^{neg}$ NK cells at baseline and on treatment, which we describe to be associated with SVR in patients with chronic HCV infection, could be due to increased proportions of dead cells in this particular NK subset. Curiously, retrospective flow cytometric analysis of their mononuclear cell samples stored in liquid nitrogen revealed that the vast majority of $CD56^{dim}/CD16^{neg}$ NK cells (mean 95%) were actually composed of dead cells. In view of this unexpected finding, Pembroke concludes that our data are not compatible with those of others (which?), being flawed by a purportedly high number of dead cells in this specific NK-cell subset. Beside any technical consideration, and even allowing for this being indeed the cause of our findings, the first comment that comes to one's mind is why would dead NK cells concentrate in SVR patients only and not in non-responders (NR) or relapsers and why would they be

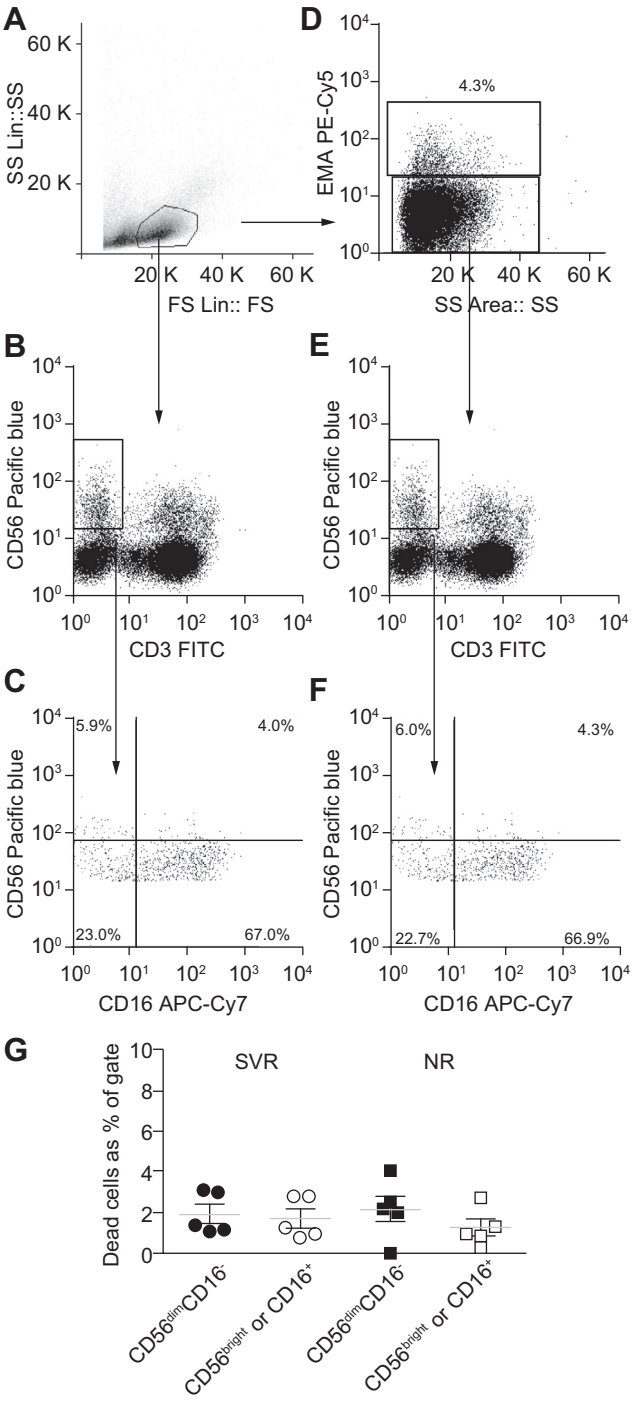

Fig. 1. Gating strategy. (A–F) Gating strategy to identify $CD56^{dim}CD16^{neg}$ NK cells in a representative SVR patient. (G) No differences between the proportions of dead cells (mean ± SEM) in the $CD56^{dim}/CD16^{neg}$ and $CD56^{bright}$ or $CD16^{pos}$ NK subpopulations in SVR and NR patients.

confined to the $CD56^{dim}/CD16^{neg}$ subset? Unfortunately, we have no plausible explanation for either question, nor do we have evidence of such a high percentage of dead cells in our preparations, particularly in the $CD56^{dim}/CD16^{neg}$ subset.

To check for possible flaws in our analysis, we also went back to our frozen samples, and examined the proportions of dead cells in the various NK-cell subsets as indicated by Pembroke. Moreover, we repeated the analysis of the prevalence of $CD56^{dim}/CD16^{neg}$ NK cells in an independent set of 10 patients, 5 SVR and 5 NR, to check whether there was any peculiarity in our original cohort of patients [1]. Fig. 1A–F shows the gating strategy to identify $CD56^{dim}CD16^{neg}$ NK cells in a representative SVR patient. Lymphocytes were gated by morphological features (A) and NK cells identified as $CD56^{pos}/CD3^{neg}$ (B) which were analyzed on the basis of CD56 and CD16 expression without excluding dead cells (C). Alternatively, dead cells were excluded by gating ethidium monoazide (EMA) positive events which represented only 4.3% of the total number of events (D). Interestingly, the proportion of $CD56^{dim}/CD16^{neg}$ NK (F) obtained from NK cells after exclusion of dead cells (E) was virtually identical to that obtained without excluding EMA^{pos} cells (C), indicating that our data were not influenced by an allegedly high concentration of dead cells in the $CD56^{dim}/CD16^{neg}$ NK subset, as suggested by Pembroke *et al.* Moreover, Fig. 1G clearly shows that there were no differences between the proportions of dead cells (mean ± SEM) in the $CD56^{dim}/CD16^{neg}$ and $CD56^{bright}$ or $CD16^{pos}$ NK subpopulations in SVR and NR patients. Finally, we were gratified to confirm that, as already reported in our manuscript, the percentages of $CD56^{dim}/CD16^{neg}$ among SVR and NR were significantly different (mean ± SEM): 21.9% ± 4.3 *vs.* 6% ± 1.1, respectively, $p = 0.007$, by including all cells, and 21.5% ± 4.2 *vs.* 5.8% ± 1.05, respectively, $p = 0.007$, after exclusion of dead cells, also in this independent set of patients.

We believe that the above strongly corroborates our recently published findings [1] and we are particularly grateful to Pembroke for allowing us to dispel any possible doubt regarding the quality of our data.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Reference

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