To the editor:

**Human B-1 cells are not preplasmablasts: analysis of microarray data and other issues**

We recently reported that human B-1 cells express the phenotype CD20+CD27-CD43 CD70-.1,2 Covens et al. now assert that B cells with this phenotype resemble plasmablasts and thus represent pre-plasmablasts. Their conclusion rests to a large extent on microarray data that they interpret as positioning B-1 cells closer to plasmablasts than to memory (or naïve) B cells.

Any similarity analysis of microarray datasets depends on several critical choices regarding: (1) how “closeness” between gene profiles is measured; (2) how many genes are considered; and (3) how genes are selected for inclusion. Covens et al chose: (1) Pearson’s correlation coefficient; (2) 100 top-ranked genes; and (3) genes ranked by plasmablast-over-memory or plasmablast-over-B-1 1-sided fold-change.

We found these choices to be unusual and we examined how the outcome may be altered with other choices. In particular, we calculated the distance (either Euclidean or Manhattan distance) to measure closeness, then varied the number of genes, and considered several other gene selection approaches. Our results clearly indicate that when distance is used to measure closeness, B-1 cells are closer to memory B cells than to plasmablasts regardless of number of

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**Figure 1. Re-analysis of publicly available microarray data reported by Covens et al.**

(A) Ratio of two Euclidean distances (averaged over 9 sample-pairs): distance between B-1 cell and memory B cell (MEM), over the distance between B-1 cell and plasmablast (PB). Six gene selection schemes are used. Red: genes are ranked by the (1-sided) plasmablast-over-memory B cell fold-changes; brown: (one-sided) plasmablast-over-B-1 cell fold-changes; blue: smaller P value from the 4-group (naive B cell, memory B cell, B-1 cell, plasmablast) ANOVA test; light blue: larger 12-sample variance; green: larger plasmablast expression level; and dark blue: random selection of genes. The x-axis is the number of genes in the gene set (in the log-scale). The dashed horizontal line separates the two situations: B-1 cells being closer to plasmablasts (above), and B-1 cells being closer to memory B cells (below). (B) Similar to (A) with Manhattan distance being used. (C) Difference of 2 Pearson correlation coefficient (cc) converted z values (averaged over 9 sample-pairs): z between B-1 cell and memory B cell, subtracts z between B-1 cell and plasmablast. The z is defined by the Fisher transformation: z = .5 log(1 + cc)/(1 − cc). The dashed horizontal line separates the situation for B-1 cells being closer to memory B cells (above) and that for B-1 cells being closer to plasmablasts (below). (D) Similar to (C) with Spearman correlation coefficient (and the corresponding z value) being used. (E-F) Scatter plot of log-expression level with the top 100 genes selected by the one-sided plasmablast-over-B-1 cells fold changes. (E) a B-1 cell (GSM1048794) (x-axis) versus a plasmablast (GSM1048797) (y-axis) for the top 100 genes selected by one-sided plasmablast-over-B-1 cell fold-change; (F) a B-1 cell (GSM1048794) (x-axis) versus a memory B cell (GSM1048791) (y-axis) for the same set of 100 genes.
genes analyzed or selection criteria for analysis (Figure 1A-B). When correlation coefficient is used to measure closeness, B-1 cells are again closer to memory B cells than to plasmablasts, regardless of number of genes analyzed, for all other gene selection methods except the 1-sided fold-change selection (Figure 1C-D). Our analysis reproduces the conclusion of Covens et al, but only for their specific choices, including the choice of using 100 genes. When the number of genes analyzed is increased beyond 200-1000 even with the 1-sided fold-change gene selection, B-1 cells are closer to memory B cells than to plasmablasts. This conclusion is alternatively confirmed by evaluating the number of genes that differ by 2-fold or more (and 0.5 or less) and P value < .05 in 2-way comparisons involving all genes examined (Table 1). Here, many more genes differ between B-1 cells and plasmablasts as compared with B-1 cells and memory B cells.

The fundamental difference between using correlation and using distance as a measure of closeness is illustrated in Figure 1E-F, which is the 100-gene set used in Figure 4B of Covens et al to show B-1 cells being closest to plasmablasts cells. Indeed, the Pearson correlation coefficient between B-1 cells and plasmablasts (0.951) is slightly larger than that between B-1 cells and memory B cells (0.892). However, the scattering of the samples is much closer to the diagonal line between B-1 and memory B cells, indicating a closer distance. If the gap between the scattering of points and the diagonal line for B-1 cells vs plasmablasts is not due to a batch effect, there is no reason to believe that the B-1 cell expression profile of these 100 genes is not closer to that of memory B cells. The 2 correlation coefficients in Figure 1E-F are not significantly different, and minor changes in detail (eg, use of 2-sided fold-change, P value, or combinations of P value and fold-change as the selection criterion; inclusion of larger number of genes; removal of a few outlier genes) could switch the order of the two.

Thus, the similarity between B-1 cells and plasmablasts suggested by Covens et al relates to a highly restricted set of analytical parameters and is contradicted by analyzing closeness according to distance rather than correlation and/or by considering more genes and/or by gene selection criteria other than the 1-sided fold-change. Our analysis of their data indicates instead that human B-1 cells are closer to memory B cells than to plasmablasts, which fits well with the memory function recently reported for mouse B-1 cells.

Other criteria by which Covens et al propose that CD20+CD27+CD43+CD70- B cells are plasmablasts include: spontaneous secretion of immunoglobulin A; induction of CD43 expression on CD43- B cells without expression of exclusionary CD69 and CD70; response to T-dependent tetanus toxoid antigen vaccination; and loss of CD20 expression by a fraction of CD20+CD27+CD43+CD70- B cells after stimulation by a Toll-like receptor agonist and cytokine cocktail. These observations are not persuasive for several reasons. B-1 cells are known to isotype switch, in mouse and human, especially to immunoglobulin A, so this property cannot determine lineage. The lack of positive controls for CD69 and CD70 makes it difficult to evaluate negative staining results, and up-regulated CD69 expression might have declined by 5 days. Further, CD43 is an activation antigen so its inducible expression, like CD5 in mouse, does not obviate its use as a lineage marker. The lack of prevaccination enzyme-linked immunospots mars interpretation of B-1 cell immunoglobulin that is known to be polyreactive. But beyond that, these authors and, separately, Westerink and colleagues, recently reported that the same CD20+CD27+CD43+ B-1 cells are responsive to pneumococcal polysaccharide vaccination, a function attributed to B-1 cells, which raises the possibility that Covens et al isolated a predominant population of B-1 cells that inadvertently contained a small number of (tetanus-responsive) non-B-1 cells. B-1 cells are known to be capable of further differentiation, including acquisition of CD138 (less than 1% of all B cells in Covens et al’s Figure 5), so induction of plasma cell phenotype is no more an argument for B-2 lineage than for B-1 lineage. Further, the gates used to identify CD20+ B cells appear to have included some CD20lo B cells that may have later, after stimulation, been counted as CD20+ and, importantly, the vast majority of B-1 cells did not change phenotype (lose CD20 and/or express CD38) after vigorous stimulation (Covens et al’s Figure 5).

In sum, the report by Covens et al does not provide substantive evidence of a plasmablast phenotype for CD20+CD27+CD43+CD69-CD70- human B cells; rather, these B cells represent a population with functional similarities to mouse B-1 cells and thus are designated human B-1 cells.

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Contribution: T.L.R. conceived the study; W.L and F.B. evaluated Covens and colleagues’ microarray data and generated the analysis shown in Table 1; W.L. generated the analysis shown in Figure 1; and W.L. and T.L.R. wrote the letter.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

Table 1. Number of differentially expressed probes between a combination of any 2 groups with a t test P value of <.05 and a 2-fold change in either direction

<table>
<thead>
<tr>
<th>Groups</th>
<th>Differentially expressed probes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1 vs memory</td>
<td>110</td>
</tr>
<tr>
<td>B1 vs naïve</td>
<td>330</td>
</tr>
<tr>
<td>B1 vs plasmablast</td>
<td>1523</td>
</tr>
<tr>
<td>Memory vs naïve</td>
<td>271</td>
</tr>
<tr>
<td>Memory vs plasmablast</td>
<td>2091</td>
</tr>
<tr>
<td>Naïve vs plasmablast</td>
<td>2667</td>
</tr>
</tbody>
</table>
Response

Extended analysis of microarray data does not contradict preplasmablast phenotype of human CD20+CD27+CD43+ cells

Li et al\textsuperscript{1} used additional approaches to analyze the microarray data presented in our article describing a preplasmablast phenotype for the proposed human B1 cells.\textsuperscript{2} Their analysis positions “B1” cells closer to memory B cells (CD19\textsuperscript{+}CD20\textsuperscript{−}CD27\textsuperscript{−}CD43\textsuperscript{−}) than to plasmablasts (CD19\textsuperscript{+}CD20\textsuperscript{−}CD27\textsuperscript{+}CD43\textsuperscript{+}), which, according to them, contradicts our conclusions.\textsuperscript{3} On the contrary, we feel that the analysis by Li et al\textsuperscript{1} supports and extends our findings. Both our analysis and theirs are compatible with the notion that the putative B1 cells have a phenotype intermediate between memory B cells and plasmablasts. In addition, the observation that surface expression of CD43 and CD27 is intermediate between that of memory B cells\textsuperscript{3,4} and plasmablasts supports our conclusions. Importantly, an independent analysis of our data demonstrates that transcriptional features of mouse B1 cells are clearly distinct from those of the proposed human B1 cells, whereas mouse and human germinal center B cells share such features.\textsuperscript{5}

Other concerns were raised by Li et al.\textsuperscript{1} We agree that B1 cells can switch to (mostly) immunoglobulin A (IgA). However, in mice, the majority of B-1 is known to secrete IgM.\textsuperscript{6} In our study, we found that IgA was the predominant isotype produced and even found similar frequencies of cells producing IgM and IgG. Rather, our data demonstrate a parallel between plasmablasts and the proposed B-1 cells.

Li et al\textsuperscript{1} suggested that tetanus toxin–specific cells in our sorted “B1” population result from a small inadvertent contamination. However, our enzyme-linked immunospot data demonstrate that if production of anti-tetanus toxin antibodies by B1 cells was due to contaminating plasmablasts, the contamination of B1 cells by plasmablasts would have accounted for up to 50\%. Moreover, such contamination would have aligned “B1” cells much closer to plasmablasts in the gene expression analysis, which is inconsistent with Li et al’s interpretation of the gene expression analysis.\textsuperscript{1} The absence of antibody production in memory B cells (both spontaneous and vaccination-induced) further underscores the purity of our sorted samples.\textsuperscript{2,7}

Li et al\textsuperscript{1} argued that differentiation of cells to plasmablasts and plasma cells does not discriminate between B-cell lineages, as we actually discussed previously.\textsuperscript{2} However, this assertion contradicts the Rohrstein group’s previous publications in which the inability of human “B1” cells to differentiate to plasmablasts was twice claimed to distinguish B-1 cells from plasmablasts.\textsuperscript{8,9}

Last, Li et al\textsuperscript{1} raised technical concerns about the absence of CD69 and CD70 expression in our stimulated B-cell cultures, pointing toward the lack of positive controls and that the 5-day timespan might have led to decreased expression. Although not mentioned in our publication, we performed parallel control experiments in which peripheral blood mononuclear cells were stimulated with R-848 and interleukin-2 and consistently observed expression of CD69 and CD70 in CD19\textsuperscript{−} lymphocytes. Before choosing day 5, we performed kinetic studies and observed a gradual increase of CD20\textsuperscript{+}CD27\textsuperscript{−}CD43\textsuperscript{+} cells from days 3 to 5 without expression of CD69 or CD70.

In conclusion, the microarray analysis performed by Li et al\textsuperscript{1} and by Mabbott and Gray\textsuperscript{3} support the concept that the putative B1 cells have a phenotype intermediate between memory B cells and plasmablasts.

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Acknowledgments: X.B. is a senior clinical investigator of the Fund for Scientific Research–Flanders.

Contribution: K.C. drafted the manuscript; and B.V., M.J., and X.B. critically revised the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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